

ROLE OF NA/K-ATPase IN BULL SPERM CAPACITATION

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By

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ABSTRACT

Sodium/potassium ATPase (Na/K-ATPase) is also a membrane signaling protein that induces capacitation in bull sperm when exposed to the highly-specific steroid hormone ouabain. Specific signalling roles of Na/K-ATPase subunit isoforms $\alpha 1$, $\alpha 3$, $\beta 1$, $\beta 2$ or $\beta 3$, which have been identified in bull sperm head plasma membrane (HPM), are unknown. The first trial hypothesized that isoforms involved in capacitation would be concentrated in HPM rafts. For the first time, rafts were isolated from fresh, uncapacitated dairy bull sperm HPM as detergent-resistant membranes that contained the marker protein flotillin-1. The ATPase isoforms $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$ and $\beta 3$ were identified in matched raft, non-raft and HPM by SDS-PAGE and immunoblotting using specific monoclonal antibodies and quantified with Image Quant Software (GE Healthcare). The $\alpha 2$, identified in HPM for the first time, and the previously-known $\alpha 1$, $\alpha 3$ and $\beta 1$, $\beta 2$, $\beta 3$ isoforms were not confined to, or enriched in, any fraction. The $\alpha 3$ and $\beta 1$ were the major isoforms in the rafts, non-raft and HPM. Rafts had unique patterns and amounts of the bands of each isoform, which could indicate their signalling role. To examine induction of tyrosine phosphorylation (Tyr-p) and capacitation, sperm were incubated with 0-100 μ M ouabain, progesterone (P4) or ouabain + P4 under capacitating conditions. Progesterone was hypothesized to act by mimicking ouabain to induce Tyr-p and/or microscopically-identified AR. Both P4 and ouabain stimulated Tyr-p of three sperm proteins, but ouabain also stimulated Tyr-p of four additional proteins and induced significantly more overall Tyr-p after 3 and 5 hours of incubation, and only ouabain induced the AR. Co-incubating sperm with ouabain plus P4 reduced ouabain-induced Tyr-p. Sperm from a subset of good fertility bulls were capacitated by ouabain and non-responsive to P4, while sperm from low-fertility bulls didn't respond to ouabain until 5 hours of incubation, and were stimulated

by P4 to produce significant Tyr-p. This suggests that P4 and ouabain may both interact with Na/K-ATPase, but ouabain significantly stimulated capacitation. The findings here provide a better understanding about the molecular mechanisms associated with capacitation and insights for developing methodology for predicting bull sperm fertility.

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LIST OF ABBREVIATIONS

AC	Adenyl cyclase
AKAP	A-kinase anchoring proteins
Akt/PKB	Protein kinase B
AR	Acrosome reaction
BSPs	Bovine seminal plasma proteins
cAMP	cyclic Adenosine Monophosphate
CBM	Caveolin binding motifs
DAG	1,2-Diacylglycerol
DLD	Dihydrolipoyl dehydrogenase
DRMs	Detergent resistant membranes
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinases
FITC-PSA	Fluorescein isothiocyanate conjugated pisum sativum agglutinin
GPI	Glycosylated phosphatidyl inositol
Grb2	Growth factor receptor-bound protein 2

GTP	Guanosine triphosphate
GST	Glutathione S-transferase
HCO ³⁻	Bicarbonate
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
TEMED	N, N, N', N'-tetramethylethylene-diamine
HPM	Head plasma membrane
HRP	Horse radish peroxidase
IP3	Inositol 1,4,5-trisphosphate
IP3R	Inositol trisphosphate receptor
LPC	L- α -Lysophosphatidylcholine
MAPK	Mitogen activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
mPR	Membrane progesterone receptor
MW	Molecular weight
Na/K-ATPase	Sodium potassium ATPase
ODF	Outer dense fiber
P4	Progesterone

PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PE	Phosphatidylethanolamine
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5 -bisphosphate
PIP3	Phosphatidylinositol 3, 4, 5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PS	Phosphatidylserine
ROS	Reactive oxygen species
Ras	Rat sarcoma protein
Raf	Rapidly accelerated fibrosarcoma
RTK	Receptor tyrosine kinases
sAC	Soluble adenylyl cyclase

SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SM	Sphingomyelin
SNARE	Soluble N-ethylmaleimide sensitive factor attachment receptor
Sos	Son of sevenless
Sp-TALP	Tyrode albumin lactate pyruvate medium
Sp-TALPH	Tyrode albumin lactate pyruvate hepes medium
Src	Sarcoma
TLCK	N- α -p-tosyl-l-lysinechloromethyl ketone
TM	Transmembrane domain
Tyr-p	Tyrosine phosphorylation
ZP	Zona pellucida

1.0 GENERAL INTRODUCTION

Artificial insemination in cattle using preserved sperm from superior quality bulls is of great importance for improving the productivity and profitability in both the dairy and beef industries. Reproductive efficiency is significantly affected by the fertility of the bull. Predicting the fertility of a bull when he is young would help producers eliminate low fertility bulls early and reduce the costs associated with housing and maintenance of these bulls. The use of semen with known fertility for insemination will reduce the inter-calving interval and number of open cows and increase productivity and profitability. Thus, it is important to develop an accurate method for predicting the fertility of bulls for both beef and dairy industry that would revolutionize the areas of artificial insemination and animal agriculture.

Fertility-related proteins in sperm cells and their signalling pathways play a significant role in determining the fertility of a bull (Ashrafzadeh et al., 2013). Mammalian sperm needs to undergo a series of biochemical and physiological changes called capacitation in the female reproductive tract before meeting the egg, in order to fertilize it (Austin, 1951; Chang, 1951). Timely capacitation of spermatozoa plays a crucial role in achieving the pregnancy of female. But the exact molecular mechanisms involved in capacitation-associated events are still under investigation. Na/K-ATPase, a well-known ion transporter protein present on the sperm plasma membrane has a vital role in supporting sperm motility (Jimenez et al., 2011). It also acts as a signalling molecule for sperm capacitation in the presence of the hormone ouabain (Thundathil et al., 2006). Progesterone (P4) is another steroid hormone which influences capacitation and acrosome reaction (AR) of mammalian sperm. Even though the exact receptor through which P4 exerts its action on mammalian spermatozoa is not known, studies on amphibian oocytes propose that Na/K-ATPase can act as a potential receptor (Morrill et al., 2005; Morrill et al., 2008). But

the exact nature of the signal(s), and how they are received is still unknown. Because Na/K-ATPase is involved in various sperm functions and its expression is reduced in abnormal sperm (Thundathil et al., 2012), it might be a potential fertility marker of sperm, and hence it is important to study the precise role of Na/K-ATPase on sperm function, its mechanism of action and the signalling cascade involved.

2.0 REVIEW OF LITERATURE

2.1 Introduction

In order to fertilize the egg, the spermatozoa should undergo various orchestrated complex reactions, starting with spermatogenesis in the testes right through sperm-zona pellucida interaction in the fallopian tube. Any defect within this multi-step process will adversely affect fertility. The sperm proteome plays a key role in determining the fertility of mammalian spermatozoa. These proteins have been categorized into structural and other functional proteins which play a key role in sperm motility, capacitation, AR and finally fertilization with oocyte. Spermatozoa need proteins with new functions to undergo various physiological and biological modifications to achieve fertilizing ability. Since spermatozoa are devoid of any major transcriptional and translational activity, peripheral proteins and post-translational modifications play a significant role in attaining sperm fertility. Different proteins are studied as fertility markers of spermatozoa in distinct species. Thus, a very good understanding of the factors influencing molecular functions of spermatozoa need to be studied in detail to accurately predict bull fertility.

2.2 Semen Production and Transport

Mammalian spermatozoa, which contribute half of the nuclear genetic material to the offspring, are highly motile haploid germ cells produced by the testis in the male. Spermatogenesis occurs within the seminiferous tubules by a series of mitotic and meiotic cell divisions (Senger, 2012). The newly formed spermatozoa which are released into the lumen of seminiferous tubules by spermiation are still immature and do not have motility and fertilizing capacity. Final maturation of spermatozoa in the male occurs during epididymal transport where interaction with epididymal fluid incorporates various molecules derived from the epididymal epithelium as well

as post-translational modifications of various endogenous proteins, leading to a series of biochemical and physiological changes resulting in the acquisition of motility and fertility potential (Gadella et al., 2008; Cornwall, 2009). During ejaculation, sperm cells are mixed with fluids from the accessory sex glands (seminal plasma) rich in various proteins, hormones, cholesterol and other biomolecules which causes some biochemical and surface modifications that facilitates spermatozoal function (Gwathmey et al., 2006).

Once the spermatozoa are deposited in the female reproductive tract, they are transported quickly through the cervix and uterus and reach the utero-tubal junction where they form a quiescent reserve, and their functional viability is maintained there for a species-specific period (Suarez and Pacey, 2006; Rodriguez-Martinez, 2007). Once the seminal plasma is removed from the sperm surface, a series of reversible biochemical and physiological changes associated with capacitation begins which is necessary for progressing to the AR and finally fertilization followed by syngamy. Fertilization is the process by which sperm from the male and egg from the female unite together to produce a genetically distinct individual. But freshly ejaculated sperm are not capable of fertilizing an oocyte (Yanagimachi, 1994) since their surface contains decapacitating factors of epididymal and/or seminal plasma origin (Fraser, 1998), comprised mainly of cholesterol and other sterols and various proteins (Davis, 1981; Yanagimachi, 1994). These are adsorbed on the sperm plasma membrane during their transport through the male reproductive tract. Sperm morphology is closely related to fertilizing potential and the proper structure and maturation of the spermatozoon is critical for successful fertilization (Al-Makhzoomi et al., 2008).

2.3 Spermatozoa

A mature mammalian spermatozoon is a highly differentiated single cell consisting of a head and tail with the entire spermatozoon covered by the plasma membrane. The head consists of a nucleus, the acrosome which covers the anterior two-thirds of the nucleus and the post-nuclear cap. The acrosome is a membrane-bound lysosome (Moreno and Alvarado, 2006) situated in between the outer nuclear membrane and the sperm plasma membrane containing the hydrolytic enzymes acrosin, hyaluronidase, zona lysine, esterases and acid hydrolases; these are required for the penetration of sperm into the zona pellucida (ZP) of the ovulated oocyte. The sperm tail which is a self-powered flagellum consists of the middle piece, principal piece, and terminal piece. The main structural component of the flagellum is the axoneme which is composed of a typical “9 + 2” microtubular pattern that extends the full length of the flagellum. The outer dense fibers (ODF) cover the axoneme both at the middle piece and the principal piece. The middle piece contains a high concentration of mitochondria surrounding the ODF and serves as the ATP source for motility, which is achieved by tail movements. The mitochondrial sheath ends at the beginning of the next portion of flagellum, the principal piece where the ODF is surrounded by a fibrous sheath. The fibrous sheath which is a characteristic of the principal piece, tapers in thickness towards the distal part and it is absent in the terminal piece. The ODF are also absent in the terminal piece. Various flagellar structures, as well as the phosphorylation status of different flagellar proteins, determine the motility pattern of sperm.

2.3.1 Sperm Plasma Membrane

The plasma membrane plays a significant role in the acquisition of motility and fertilizing capacity of spermatozoa in addition to its role in controlling trans-membrane transport of various

ions (Cornwall, 2009; Gadella and Boerke, 2016). The plasma membrane surrounds the entire sperm cell and contains a high proportion of polyunsaturated phospholipids, other lipids including cholesterol and proteins organised into discrete domains on the sperm head and tail (Gadella et al., 2008). These biomolecules are not randomly arranged, but instead are maintained in a typical asymmetry of specific molecules which is required for the proper functioning of spermatozoa (Rana et al., 1993; Gadella and Harrison, 2000). The membrane lipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly located on the inner leaflet of the plasma membrane while phosphatidylcholine (PC) and sphingomyelin (SM) are more concentrated in the outer leaflet. Lateral diffusion of various membrane lipids and proteins, unless prevented by cytoskeletal barriers, as well as the trans-bilayer movement of these lipids and proteins mediated by different transferases like flippases, floppases, and scramblases are possible on the plasma membrane (Contreras et al., 2010). These movements are greatly influenced by the fluidity of the membrane.

As in somatic cells, the plasma membrane of spermatozoa is also regionalized/compartmentalized into discrete domains and microdomains, including lipid rafts (Kawano et al., 2011; Boerke et al., 2014; Gadella and Luna, 2014). These membrane domains are highly dynamic in nature and the surface properties of plasma membrane vary throughout the transit of sperm to reach the ova, in ways that are essential for sperm maturation and fertilization (Kawano et al., 2011). Within the female reproductive tract, the sperm plasma membrane is remodelled, including re-positioning of both protein and lipid components during specific events including capacitation, AR and binding to the oocyte (Gadella and Boerke, 2016). This membrane remodelling occurs mainly in the head plasma membrane (HPM) where the sperm first interacts with the egg, and it is important for various physiological functions of sperm (Caballero et al., 2012)

2.4 Raft

In somatic cells including cardiac cells, signalling platforms involve the specific membrane rafts which regulate various cellular functions. Rafts are discrete membrane domains formed by the aggregation of different lipids mainly sphingomyelins and cholesterol which are packed together along with multiple signalling proteins and glycosylated phosphatidyl inositol (GPI) anchored proteins to form a liquid-ordered microdomain (Simone and Toomre, 2000; Simons and Sampaio, 2011). Rafts house various signalling molecules such as sarcoma (Src) family kinases, G proteins, mitogen activated protein kinases (MAPKs), protein kinase C (PKC), phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K) and some enzymes like receptor tyrosine kinases (RTK) including receptors for epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF; Simons and Toomre, 2000; Xie, 2003; Chini and Parenti, 2004; Calder and Yaqoob, 2007; Jahn et al., 2009). Co-localization of several signalling components into a raft enhances molecular interactions making signalling more efficient (Pike, 2006). The side chains of the phospholipids present on the raft are usually highly enriched in saturated fatty acids. Raft domains are also enriched with cholesterol. Because of the presence of cholesterol and saturated fatty acids which are able to pack closely, rafts form a liquid-ordered microdomain, segregated from the remaining more fluid membrane. Due to their structural characteristics, rafts are insoluble in non-ionic detergents like Triton X-100 at low temperatures and can be isolated as detergent resistant membranes (DRMs) by density gradient floatation of homogenized cellular materials (Brown and Rose, 1992; Kawano et al., 2011). Rafts are heterogeneous (Simons and Sampaio, 2011), but certain molecules like GPI-linked protein CD59, the ganglioside GM1, and other proteins like flotillin-1, flotillin-2 and caveolin-1 are enriched in all kinds of rafts and have been used as raft markers (Cross, 2004). Even though the rafts have limited mobility in the plane of the

membrane, loss of membrane asymmetry or modification of raft proteins like phosphorylation which increases the number of protein-protein interaction induces the coalescence of various rafts into large membrane domains mediated by actin cytoskeleton (Simons and Sampaio, 2011). Because of the highly-ordered arrangement, movement of molecules are restricted within the raft. But signalling associated changes induce the movement of various molecules especially receptors like EGFR or other downstream signalling molecules in and out between the raft and non-raft fractions (Abulrob et al., 2004; Schley et al., 2007; Lozano et al., 2016). Other than signalling induced changes, altered lipid content of the raft either by cholesterol efflux or by hydrolysis of other lipids within the raft may also influence the movement of proteins between DRMs and the bulk of plasma membrane (Rogers et al., 2010; Corsetto et al., 2012).

Caveolae are a subset of specialized rafts enriched with the integral membrane protein caveolin and are seen as flask-shaped invaginations on the plasma membrane (Thomas and Smart, 2008). Caveolins can tightly bind to cholesterol, and so aggregation of caveolins on the plasma membrane forces the outer leaflet of the lipid bilayer to curve inward forming caveolae on the cell surface. Caveolae are most abundant in adipocytes, smooth muscles, and endothelia (Stan, 2005). Caveolin has three isoforms, Caveolin 1-3, of which caveolin-1 is the most abundant one and is present in almost all the cells excluding striated muscle. Caveolin-2 colocalizes with caveolin-1 but cannot form caveolae by itself. Caveolin-3 is muscle-specific caveolin and is mainly located in the striated muscles. Caveolin-1 can act as a scaffold protein binding to different signalling molecules and recruit them to a signalling platform to form a signalling complex (Parton et al., 2006). So, like other rafts, caveolae house various signalling molecules together and act as a signalling platform. Other than the signalling, functions of caveolae also include regulation of endocytosis, transcytosis, lipid homeostasis and mechanoprotection of cells (Cheng and Nichols, 2016).

Rafts including caveolae have been identified and isolated from the spermatozoa of various mammalian species (mouse, guinea pig: Travis et al., 2001; human: Cross, 2004; boar: Khalil et al., 2006; bull: Post et al., 2010; ram: Colas et al., 2012; Rajamanickam et al., 2017). In most of the experiments, rafts were isolated from the whole sperm. These lipid rafts play a significant role regulating signal transduction pathways associated with acquiring sperm maturational changes capacitation, hyperactivation and AR (Kawano et al., 2011; Boerke et al., 2014; Gadella and Luna, 2014). Other than the signalling molecules, lipid rafts in sperm house various proteins involved in the egg-zona binding such as CD59, fertilin- β , AQN-3/spermadhesin and P47/SED-1 suggesting a major role of the raft for egg zona binding (van Gestel et al., 2005; Tanphaichitr et al., 2007).

2.5 Capacitation

In 1951 Austin and Chang separately discovered that the ejaculated spermatozoa must reside in the female reproductive tract for a species-dependent time in order to acquire fertilizing ability. During this period, a series of functional, biochemical and physiological cellular modifications called capacitation occur. Capacitation is initiated by signals that the sperm receives in the female reproductive tract which cause the sperm to rearrange its membrane and begin specific internal reactions. Various factors in the tubal fluids of female reproductive tract trigger capacitation (Aquila and De Amicis, 2014), but the exact nature of the signal(s), and how they are received, is unknown. Capacitation can be induced in ejaculated sperm *in vitro* under a variety of conditions in defined media containing species-specific energy substrates, a protein source (usually serum albumin), sodium bicarbonate (NaHCO_3), and calcium (Ca^{2+}) which mimic the electrolyte composition of the oviductal fluid (Harrison, 1996; Visconti et al., 1998). Capacitation of bovine sperm can be stimulated *in vitro* by using capacitation-inducing agents like heparin (Parrish et al., 1988) and ouabain (Thundathil et al., 2006) in the medium. In sperm, the ouabain

stimulated pathway is different from the heparin-stimulated pathway. Heparin induces capacitation primarily by stimulating the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway (Parrish et al., 1994; Parrish, 2014) whereas ouabain induces sperm capacitation through interactions with Na/K-ATPase, resulting in the activation of Src and phosphorylation of serine and tyrosine residues to ultimately activate extracellular signal-regulated kinases1/2 (ERK1/2) to stimulate bull sperm capacitation (Rajamanickam et al., 2017).

2.5.1 Capacitation-associated Molecular Changes

Capacitation-associated changes remodel the sperm plasma membrane and activate specific ion channels and transporters located on the sperm plasma membrane which drive various ion fluxes in spermatozoa. The major ionic changes include increases in intracellular concentration of Ca^{2+} , bicarbonate (HCO_3^-) and the production of reactive oxygen species (ROS). Modified intracellular ionic concentrations increase the fluidity which finally leads to depolarization of sperm plasma membrane (Flesch and Gadella, 2000). Changes due to membrane modification finally activate a cascade of phosphorylation events resulting in increased tyrosine phosphorylation (Tyr-p) of sperm proteins which is a hallmark event of capacitation (Naz and Rajesh, 2004; Thundathil et al., 2006; Salicioni et al., 2007; Newton et al., 2010; Jagan Mohanarao and Atreja, 2011). Capacitation of spermatozoa results in hyperactivation and AR.

2.5.1.1 Bicarbonate

Bicarbonate is one of the critical molecules involved in capacitation (Wang et al., 2003, Battistone et al., 2013, Macías-García et al., 2015). Immediately after ejaculation, the spermatozoa are exposed to seminal plasma with higher HCO_3^- levels than in the epididymis. This high HCO_3^-

level induces a rapid HCO_3^- influx and an associated increase in intracellular pH in the spermatozoa. The HCO_3^- enters the sperm through $\text{Na}^+/\text{HCO}_3^-$ cotransporter (Demarco et al., 2003). Increased HCO_3^- along with increased pH activate soluble adenylyl cyclases (sACs) which then increase the production of cAMP and, subsequently, the activation of PKA, one of the central signalling pathways associated with Tyr-p of proteins and capacitation events on mammalian sperm. Incubation of dog sperm in a HCO_3^- -free medium inhibits capacitation, but addition of HCO_3^- to the medium induces capacitation (Steckler et al., 2015)

2.5.1.2 Calcium

Calcium is another central molecule involved in regulating capacitation. Capacitation is associated with an increase in intracellular Ca^{2+} concentration. Bicarbonate and pH-mediated structural changes, as well as the hyperpolarization of the membrane, induce the opening of various calcium ion channels mainly CatSper and other voltage-gated channels through which the Ca^{2+} influx occurs (Navarrete et al., 2015). Calcium acts in parallel with HCO_3^- , inducing capacitation through stimulation of adenylyl cyclases (AC). Increased cytosolic Ca^{2+} is also necessary to activate additional sperm signalling events for hyperactivation and AR. Calcium is also necessary for the aggregation of rafts towards the apical plasma membrane (Leahy and Gadella, 2015), and indeed low Ca^{2+} triggered a random distribution of membrane proteins over the entire head surface of boar sperm (Aguas and Da Silva 1983).

2.5.1.3 Reactive Oxygen Species (ROS)

Another molecular event associated with capacitation of mammalian sperm is the mild increase in ROS production during early stages of capacitation (de Lamirande et al., 1997a). Bicarbonate supports an optimal level of production of ROS. The effect of ROS on capacitation is

dose dependent; at physiological concentrations it induces cAMP production, Tyr-p of proteins and capacitation of mammalian sperm (Lewis and Aitken, 2001; Baumber et al., 2003; Rivlin, 2004; Awda et al., 2009; Aitken et al., 2012; Du Plessis et al., 2015) while higher doses are toxic as they can destroy various unsaturated fatty acids in the sperm plasma membrane and inhibit zona-sperm interaction (Aitken and Clarkson, 1989; Jain, 2010). Physiological concentrations of ROS are also important for the cholesterol efflux during capacitation, as ROS can oxidize cholesterol to form oxysterols that can activate the sterol transporter proteins to promote cholesterol efflux (Brouwers et al., 2011, Boerke et al., 2013). ROS effects may be species-specific, since horse sperm in standard capacitating conditions neither increase ROS production nor cholesterol efflux (Macías-García et al., 2015).

2.5.1.4 Cholesterol Efflux

One of the significant changes normally seen during capacitation is the removal of cholesterol from the sperm plasma membrane induced by cholesterol acceptors such as albumin and other high-density lipoproteins present in the female reproductive tract (Davis, 1981; Visconti et al., 2002) and subsequent decrease in the cholesterol content of sperm plasma membrane. Hamster spermatozoa incubated in a capacitation medium devoid of albumin neither hyperactivated nor capacitated (Noguchi et al., 2008). Cholesterol which gets deposited during epididymal maturation (Saez et al., 2011) stabilizes the sperm plasma membrane during their initial transit through the female reproductive tract. Bicarbonate has been reported as one of the critical factors for regulating the redistribution of cholesterol on sperm plasma membrane. Increased HCO_3^- has been seen associated with the activation of scramblase enzyme on the plasma membrane which destroys membrane asymmetry and subsequent membrane architecture (Gadella and Harrison, 2000). These membrane structural modifications increase the availability of

cholesterol to cholesterol acceptors like albumin (Salicioni et al., 2007; Visconti, 2009). The absence of HCO_3^- in the capacitation medium results in an unaltered level of cholesterol even in the presence of albumin (Leahy and Gadella, 2015). Capacitation-dependent activation of reverse sterol transporters specifically lifts free sterols out of the plasma membrane lipid bilayer and encourages the movement of cholesterol out from the sperm plasma membrane towards the acceptor molecules (Aitken and Nixon, 2013; Boerke et al., 2013; Gadella and Boerke, 2016). Non-albumin cholesterol binding agents such methyl- β -cyclodextrin (MBCD) can also induce capacitation of mammalian sperm and enhance the fertility in various species including mouse and stallion (Takeo et al., 2008; Bromfield et al., 2014; Yoshimoto et al., 2017).

2.5.1.5 Tyrosine Phosphorylation

One of the critical molecular responses associated with capacitation is Tyr-p of sperm protein (Gur and Breitbart, 2008; Signorelli et al., 2012; Campbell et al., 2013). Since the spermatozoon is a transcriptionally less active cell, post-translational modifications such as Tyr-p are the primary means of modifying protein function to control cell activities such as fertilization (Brohi and Huo, 2017). Following the stimuli for capacitation, various signalling pathways including cAMP/PKA pathway or ERK1/2 pathway are activated, phosphorylating serine/threonine residues on various protein kinases to activate them thereby stimulating the Tyr-p of multiple proteins. Tyr-p of proteins occurs during the later stages of capacitation on a different timescale. The proteins which get phosphorylated during mammalian sperm capacitation are mostly cytoskeletal structural proteins, metabolic enzymes or ion channel proteins, activation of which are necessary for the sperm to achieve hyperactive motility, undergo AR and binding to the zona pellucida of the egg for fertilization. Mass-spectrometry based protein analysis provides a

comprehensive approach to identify and characterize numerous tyrosine phosphorylated proteins in capacitated sperm.

One of the major proteins that is phosphorylated during mammalian sperm capacitation is A-kinase anchoring proteins (AKAPs). AKAPs contain PKA anchoring domains, and they also form complexes with other signalling molecules including various kinase and phosphatases (Miki and Eddy, 1999; Skroblin et al., 2010; Welch et al., 2010). Among different AKAPs, AKAP4 is a major fibrous sheath protein in mammalian sperm that acts as a switch molecule between the cAMP/ PKA and PKC/ERK1/2 pathways. Regulation of both pathways are essential for regulating hyperactive motility of sperm (Rahamim Ben-Navi et al., 2016). Capacitation-associated Tyr-p of AKAP4 occurs in sperm from cattle (Jagan Mohanarao and Atreja, 2011, Byrne et al., 2012), human (Carrera et al., 1996; Ficarro et al., 2003; Rahamim Ben-Navi et al., 2016), mouse (Johnson et al., 1997; Moss et al., 1999, Jivan A et al., 2009) and hamster (Jha and Shivaji, 2002; Kota et al., 2009). Deletion of AKAP4 results in shorter sperm flagellum and lack of proper fibrous sheath formation causing loss of motility and subsequent infertility (Miki et al., 2002).

Tektins are another group of cytoskeletal proteins which are phosphorylated during capacitation (Hamster: Kota et al., 2009, Mariappa et al., 2010; Buffalo: Jagan Mohanarao and Atreja, 2011). Tektins form the axoneme of the mammalian sperm tail and are involved in sperm motility. Male mice null for TEKT3 produce sperm with reduced motility and increased flagellar structural defects (Roy and Atreja, 2009). Actin is another critical cytoskeletal structural protein which is present on the equatorial, post acrosomal and tail regions of mammalian sperm. Tyr-p induced actin polymerization is necessary for regulating hyperactivated sperm motility. Inhibition of Tyr-p prevents actin polymerization and capacitation (Brener et al., 2003). Other sperm flagellar proteins that get phosphorylated during capacitation are major fibrous sheath protein (82 kDa),

outer dense fiber protein 2 (55 kDa) and outer dense fiber of sperm tails 2 (84 kDa) which are all involved in the acquisition of hyperactive motility and are required for the spermatozoa to penetrate the ZP of the oocyte and finally fertilize it (Nassar et al., 1999; Jagan Mohanarao and Atreja, 2011).

Various voltage-dependent anion channels including voltage-gated sodium channels (Chauhan et al., 2018) as well as molecules like calcium-binding tyrosine phosphorylation-regulated protein are also reported to be phosphorylated during the capacitation process in human, mouse and bovine sperm (Naaby et al., 2002; Ficarro et al., 2003; Arcelay et al., 2008; Jagan Mohanarao and Atreja, 2011; Chauhan et al., 2018). Phosphorylation of these ion channel proteins plays a critical role in maintaining the proper ionic balance of sperm which is closely related to fertility. Phosphatases are a group of enzymes which cleave the phosphoryl group from the protein and dephosphorylate them, and combined action of various kinases and phosphatases play a critical role in attaining the capacitated state of mammalian sperm. Capacitation-associated Tyr-p of serine/threonine- protein phosphatase gamma (PPI γ) was demonstrated in buffalo sperm and is essential for regulating hyperactive motility of sperm (Jagan Mohanarao and Atreja, 2011).

Glutathione S-transferase (GST) is a detoxifying enzyme which maintains the oxidative balance of the spermatozoa. GST is located in the principal piece and end piece of the sperm flagellum. Tyr-p of GST has been reported in various species associated with capacitation (buffalo: Jagan Mohanarao and Atreja, 2011; Kumar et al., 2014; mouse: Arcelay et al., 2008; hamster: Ashrafzadeh et al., 2013). Tyrosine phosphorylated GST shows significantly higher activity compared with their dephosphorylated forms in buffalo sperm (Kumar et al., 2014). Other proteins that exhibits capacitation dependent Tyr-p are the post-pyruvate metabolic enzymes pyruvate dehydrogenase and dihydrolipoyl dehydrogenase (DLD) in bovine, hamster, and buffalo (Mitra

and Shivaji, 2004; Jagan Mohanarao and Atreja, 2011). Other proteins which get phosphorylated during mammalian capacitation include ubiquinone, the component of the electron transport chain (hamster: Kota et al., 2009; bull: Jagan Mohanarao and Atreja, 2011), ERK1/2 of MAPK signalling pathway (Rajamanickam et al., 2017), membrane metallo-endopeptidase- like 1, 3-oxoacid CoA transferase 2 (Jagan Mohanarao and Atreja, 2011) and various heat shock proteins (HSP-90, 70 and 60) (Ecroyd et al., 2003; Mitchell et al., 2007; Cole and Meyers, 2011). Phosphorylation of these proteins are required for regulating sperm capacitation, hyperactive motility and AR, which are required for the spermatozoon to reach, bind, penetrate and fuse with the oocyte.

2.5.2 Capacitation-associated Membrane Changes

Capacitation results in the removal of decapacitating factors from the sperm surface. Decapacitating factors include various GPI-anchored proteins like CD52 and CD55 which get adsorbed on to the sperm surface during the transit through the male reproductive tract (Boerke et al., 2014). Removal of these decapacitating factors from the sperm surface results in the exposure of molecules which are responsible for the interaction of spermatozoa with the oocyte resulting in a functionally viable spermatozoon (de Lamirande et al., 1997b; Senger, 2012). Only capacitated spermatozoa can undergo subsequent AR and fertilization.

Capacitation is associated with loss of membrane asymmetry triggered by the influx of HCO_3^- into the sperm cell and the associated activation of the cAMP-PKA pathway. Scramblase is an enzyme that is involved in moving four-phospholipid species (PC, SM, PE and PS) in both directions (inward and outward) across the membrane lipid bilayer. Activation of scramblase activity induced by HCO_3^- increases the trans-bilayer movement of phospholipids and tends to eliminate the asymmetric distribution of these phospholipids across the membranes (Contreras et

al., 2010). Capacitation-associated cholesterol removal from the plasma membrane potentiate scramblase activity.

Capacitation-associated cholesterol efflux occurs mainly from the non-raft region of plasma membrane while the raft cholesterol stays, which means that a greater percentage of the total membrane cholesterol is in the rafts following capacitation (Khalil et al., 2006). Capacitation-associated changes alter the raft composition; the detergent-resistant fractions (raft portions) of membranes from capacitated sperm are found at a lighter density than those from non-capacitated sperm, reflecting a capacitation-dependent change in the composition of rafts (Thaler et al., 2006). Various proteins move in and out of the raft domain during sperm maturation and capacitation. In human sperm, the proteins GM1 and CD59 move out of the raft to the non-raft fraction during capacitation (Cross, 2004). Cholesterol efflux lowers the cholesterol:phospholipid ratio of the sperm plasma membrane and this altered ratio induces the remodelling of membrane lipids and phospholipids which efficiently fluidizes the plasma membrane. All these membrane modifications precede hyperpolarization of sperm plasma membrane and activation of the various signalling pathway for the progression of capacitation and probably facilitate membrane fusion during the AR (Primakoff and Myles, 2002; Martínez-López et al., 2009).

Aggregation of rafts during the membrane reorganization permits the assembly of various protein complexes into a new environment, likely contributing to the disruption of the pre-capacitation signalling and activation of new signalling pathways. Coalescence of rafts also brings various proteins involved in AR and zona binding proteins such as fertilin-beta, AQN-3/spermadhesin and P47/SED-1, carbonyl reductase and potentially others together into one big platform on the apical plasma membrane which enhances acrosomal exocytosis, and binding of sperm with ZP for successful fertilization (Khalil et al., 2006; Gadella and Luna, 2014). The apical

plasma membrane isolated from capacitated pig sperm, enriched in raft aggregates can bind to solubilized pig ZP glycoproteins, in particular, ZP3 (Khalil et al., 2006). Thus, the cholesterol efflux causes remodelling of membrane lipids and phospholipids, increases the fluidity of the plasma membrane and modifies the activity of various transmembrane proteins associated with acquiring the fertilizing ability of sperm (Gadella and Luna, 2014; Nicolson, 2014). These functional membrane modifications occur mainly in the sperm HPM and are important for sperm-ZP binding, AR and membrane fusion during fertilization (Caballero et al., 2012; Kongmanas et al., 2015).

2.6 Hyperactivation

During capacitation, initiated in the female reproductive tract after the release of decapacitating factors from the sperm surface, spermatozoa acquire a hyperactive type of motility characterized by non-progressive, high amplitude, whiplash-like movement (Yanagimachi, 1994). The possible biochemistry for hyperactivation is progesterone (P4)-mediated (Sagare-Patil et al., 2013; Smith et al., 2013) or cAMP-mediated (Alasmari et al., 2013) release of Ca^{2+} from intracellular calcium stores by influencing a sperm-specific flagellar calcium channel during capacitation. This calcium interacts with the axoneme of the flagellum to induce hyperactivated motility. Hyperactivation is crucial to fertilizing capacity of sperm as it helps the sperm to detach from the oviductal epithelium and enhance its penetration through the ZP (Suarez, 2008).

2.7 Acrosome Reaction

After attaining hyperactivated motility, capacitated spermatozoa are primed to undergo the AR in response to its attachment to the ZP protein (Gadella et al., 2008; Senger, 2012). Lipid rafts on the sperm surface play a crucial role in sperm-zona binding and the zona-induced AR.

Acrosomal exocytosis is regulated mainly by specific members of the soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) protein family (syntaxin1 and 2, and the v-SNARE VAMP). Following capacitation, these SNARE proteins migrate into the lipid rafts and cluster in the apical ridge, the exact area where sperm-zona binding and subsequent acrosomal exocytosis occurs. There are two zona binding sites on the HPM: the ‘primary zona binding region’ is responsible for binding of spermatozoa to the ZP and the ‘AR promoting ligand’ is responsible for signal transduction to initiate the AR; both are exposed during capacitation (Gupta and Bhandari, 2011). When the AR promoting ligand binds to the ZP protein on the surface of the oocyte, it initiates the irreversible changes of the AR, during which the HPM becomes closely apposed to, and then fuses with, the underlying outer acrosomal membrane. The fused membranes subsequently slough off, releasing the hydrolytic acrosomal enzymes such as hyaluronidase and acrosin and these enzymes digest the ZP and facilitate the passage of sperm through the ZP to fuse with the oocyte (Senger, 2012).

2.8 *In Vitro* Capacitation Inducing Agents

During *in vivo* fertilization, capacitation and the AR occur naturally to ejaculated spermatozoa in the female tract. Sperm from many species, whether ejaculated or obtained prior to ejaculation, can be induced to undergo capacitation and the AR in the laboratory under controlled conditions. For bull sperm, addition of various capacitation inducing agents like heparin, ouabain and P4 to the capacitation medium can enhance the *in vitro* capacitation (Parrish et al., 1988; Lukoseviciute et al., 2004; Thundathil et al., 2006). Although the appropriate conditions often vary somewhat from species to species, the ability to induce these changes *in vitro* have greatly enhanced the understanding of capacitation.

2.8.1 Heparin

Heparin is one of the commonly used *in vitro* capacitation inducing agent for bull sperm and is found naturally the bovine female reproductive tract (Lee and Ax, 1984). Heparin interacts with various bovine seminal plasma proteins (BSPs) on the sperm surface (Chandonnet et al., 1990). These BSPs get adsorbed on to the sperm surface during ejaculation (Manjunath and Thérien, 2002). The BSPs include BSP-A1, BSP-A2 and BSP-A3, which interact with both cholesterol and phospholipids in the sperm plasma membrane and protect the sperm from premature capacitation during the early stages of transport in the female reproductive tract (Gwathmey et al., 2006). Heparin binds with BSPs and induces the loss of BSPs from sperm surface which leads to efflux of membrane cholesterol and subsequent loss of membrane architecture. For heparin to induce capacitation, appropriate concentrations of HCO_3^- and Ca^{2+} are necessary in the capacitation medium (Parrish et al., 1988). The major intracellular mechanism triggered by heparin during capacitation in bovine spermatozoa is the activation of the cAMP/PKA pathway which causes the Tyr-p of various sperm proteins.

2.8.2 Ouabain

Ouabain is an endogenous cardiac glycoside that is also an endogenous steroid hormone found naturally in the mammals (Schoner, 2002). Ouabain is synthesized by the adrenal glands and the hypothalamic neurons (supraoptic and paraventricular nucleus) in mammals and the central nervous system regulate its release in to the blood stream (Schoner and Scheiner- Bobis, 2007). The precursors of endogenous ouabain in mammals are pregnenolone and progesterone (Hamlyn et al., 1998). Structurally ouabain contains a steroid core, a sugar moiety (Rhamnose) in the 3 position, a lactone ring in the 20 position and hydroxyl groups on carbons 1, 5, 11, 14 and 21 of

the steroid nucleus (Ogawa Haruo et al., 2009). Endogenous ouabain is found in blood plasma and is also present in nanomolar concentrations in bovine semen and vaginal fluids (Daniel et al., 2010; Chen et al., 2015). Ouabain at low concentration (μM - nM) acts as a specific agonist of Na/K-ATPase, a widely distributed membrane-bound protein in living cells, binding with very high affinity to a specific ouabain binding site, while high concentrations of ouabain (mM) inhibit the enzyme action of Na/K-ATPase (Wang et al., 2004; Liu and Askari, 2006; Tian et al., 2006; Schoner and Scheiner-Bobis, 2007; Reinhard et al., 2013). The steroid moiety of ouabain facilitates its binding with Na/K-ATPase, and the lactone ring contributes the inhibitory effect that ouabain has on Na/K-ATPase enzyme activity (Manunta et al., 2001; Cornelius et al., 2013). In contrast to ouabain's inhibition of the ion transport function of Na/K-ATPase, the nontoxic dose of ouabain can induce signal transduction by Na/K-ATPase, which activates Tyr-p of plasma membrane or intracellular proteins without disturbing the ion transport function of Na/K-ATPase in different cell types including cardiac myocytes and sperm (Liu et al., 2000; Wang et al., 2004; Liu and Askari, 2006; Thundathil et al., 2006; Tian et al., 2006; Reinhard et al., 2013, Xie et al., 2015; Peng et al., 2016).

2.9 Na/K-ATPase

Na/K-ATPase is a heterodimeric, amphipathic membrane protein belonging to the P-type adenosine triphosphatase superfamily (Kaplan, 2002; Morth et al., 2007). It is found in many cell types and has two distinct functions, acting as an enzyme that transports ions across the membranes of many cell types, and as an inducer of internal cell signalling. It consists of two main polypeptide subunits called alpha (α) and beta (β). The kinetic properties of Na/K ATPase are also regulated by an auxiliary protein belonging to the FXYD family called γ subunit (Kaplan, 2002; Geering,

2006). Na/K-ATPase is associated with caveolae in the outer leaflet of the plasma membrane of pig kidney cells (Wang et al., 2004).

2.9.1 Structure

The α subunit, the catalytic subunit of the sodium pump, consists of 10 transmembrane helices (TM1-TM10) composed of approximately 1000 amino acid residues with a molecular weight of about 110 kDa (Kaplan, 2002; Morth et al., 2007). This α subunit has four known isoforms, namely $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$ (Geering, 2008). This subunit is responsible for the ability of the Na/K-ATPase to hydrolyze ATP and undergo the conformational changes required for the pump to transport Na^+ and K^+ . Moreover, the α subunit contains the binding site for ouabain that specifically inhibits the enzymatic function and stimulates the signalling task of Na/K-ATPase (Kaplan, 2002). The β binding with α is critical for ouabain receptivity and enzyme action of Na/K-ATPase. Two ouabain binding sites have been reported in the amphibian oocyte, a low-affinity binding site located between transmembrane regions (TM) 1 and TM2 and a high-affinity ouabain binding site between TM4 and TM6 which differ by only a few amino acids (Morrill et al., 2008). The low-affinity ouabain binding site has structural similarity with steroid receptors and can bind to steroids with a planar configuration such as progesterone, dehydroepiandrosterone, 11-hydroxycortisol and 18-hydroxy-11-deoxycorticosterone (Secombe et al., 1989). Ouabain binding characteristics and affinity for ouabain differ among α isoforms (Pierre et al., 2008). Among different α isoforms, ouabain has a stronger affinity for $\alpha 3$ than for $\alpha 2$ or $\alpha 1$ in mammalian cells (O'Brien et al., 1993). Thus, significantly lower concentrations of ouabain are needed to activate signalling with $\alpha 3$ than $\alpha 1$ (Pierre et al., 2008).

The β subunit is a single membrane-spanning unit made up of 370 amino acids, mainly located on the exterior leaflet of the plasma membrane (Kaplan, 2002; Morth et al., 2007). The β subunit is a heavily glycosylated polypeptide with a molecular weight of about 55 kDa. This subunit has three isoforms namely $\beta 1$, $\beta 2$ and $\beta 3$ (Geering, 2008). The β subunit facilitates the proper insertion of α subunits into the membrane during synthesis of Na/K-ATPase and affects the functional properties of the α subunit during active transport (Geering, 2008). The β subunit also has a significant role in signalling action of Na/K-ATPase. For the Na/K-ATPase to function as a signal transducer, the α subunit should be paired with the β subunit (Liu and Askari, 2006). Among β isoforms, the $\beta 1$ is widely accepted as the signalling partner with $\alpha 1/\alpha 3$ in ouabain-mediated signalling (Guerrero et al., 2001; Pierre et al., 2008). In addition to supporting α for proper ATPase function, β especially $\beta 1$ plays a critical role in cell adhesion (Vagin et al., 2012).

FXYP proteins are also referred to as the γ subunit of Na/K-ATPase and are a family of small regulatory proteins. Seven homologous members of this family have been discovered in mammals (Geering, 2006). These proteins have a single transmembrane segment with an extracellular N terminus and cytoplasmic C terminus. The total number of amino acids varies from 61-178 between different members of FXYP proteins. The interaction of Na/K-ATPase with FXYP proteins is not necessary for the expression of Na/K-ATPase nor does it produce a change in the expression of this enzyme, but it has a tissue and isoform-specific regulatory effect on the kinetic function of Na/K-ATPase for adapting to the physiological need of the tissue (Geering, 2006). These proteins are seen in close proximity of the ninth transmembrane domain (TM9) of the α subunit of Na/K-ATPase, and this TM9 is involved in the interaction of Na/K-ATPase with FXYP proteins (Mishra et al., 2011).

The Na/K-ATPase functions as a dimer ($\alpha\beta$) or double dimer ($(\alpha\beta)_2$) of α and β subunits (Linnertz et al., 1998). The existence of four α and three β isoforms suggests the possibility of the occurrence of different combinations of these subunits forming different isozymes of Na/K-ATPase. Each Na/K-ATPase isozyme shows different functional properties and different affinities to their substrates depending on the specific subunit combination (Blanco, 1998). These different isoforms of α and β subunits are encoded by separate genes. (Blanco, 1998). These isoforms are tissue-specific, cell-specific and have distinct functions. The α_1 and β_1 are broadly distributed in most of the cells including spermatozoa (Hickey and Buhr, 2012) and $\alpha_1\beta_1$ is the major isozyme of Na/K-ATPase which maintains the basic Na^+ and K^+ concentrations in living cells (Liu and Askari, 2006). The α_2 isoform is found in adipocytes, muscle, heart, and brain (Blanco, 2005), and α_3 is present in nervous tissues (Dobretsov, 2005) and sperm (Hickey and Buhr, 2012). The α_4 isoform is present in testis and sperm and show the most restricted appearance (Woo et al., 2000; Newton et al., 2010; McDermott et al., 2015). The β_2 isoform is found in skeletal muscle, pineal gland, and nervous tissues, whereas β_3 is present in testis, retina, liver, and lung (Tokhtaeva, 2012). Both β_2 and β_3 are also present on sperm (Hickey and Buhr, 2012).

Na/K-ATPase subunit isoforms are detected both in the raft/caveolae and non-raft part of the cell membrane in various mammalian cells including cardiac myocytes, renal epithelial cells brain cells and sperm cells (Liu and Askari, 2006; Liu et al., 2011; de Juan-Sanz et al., 2013; Rajamanickam et al., 2017). The surrounding membrane environment greatly influences the activity of Na/K-ATPase. The lipid (Wu et al., 2001; Turner et al., 2005; Hickey and Buhr, 2011) as well as protein composition (Therien and Blostein, 2000) of the surrounding membrane modulate activity of Na/K-ATPase directly or indirectly with a more fluid membrane supporting greater enzyme activity (Sutherland et al., 1988).

2.9.2 Na/K-ATPase Ion Transport Function

The enzymatic activity of Na/K-ATPase maintains the Na^+ and K^+ concentrations across the plasma membrane, which is essential for maintaining the resting membrane potential of the cell, its osmotic stability, pH and also for secondary transport of other ions, amino acids and glucose across the plasma membrane. The sodium pump is an energy driven active transport which hydrolyzes ATP to produce energy to transport three Na^+ out and two K^+ ions into the cell thus maintaining osmotic balance of living cells for their normal functioning (Kaplan, 2002). Na/K-ATPase in both raft and non-raft fractions of the cell membrane maintain their ion transport function (Liu et al., 2011). The activity of Na/K-ATPase is also influenced by circulating endogenous inhibitors like ouabain, other steroid hormones including corticosteroids, and peptide hormone mediators such as protein kinases and phosphatases, cAMP etc. (Ewart and Klip, 1995). Ion transport activity of Na/K-ATPase is also influenced by its specific agonist ouabain. The enzyme action of Na/K-ATPase that drives ion transport is inhibited by high concentrations of ouabain (milli-molar) but stimulated by low nanomolar concentrations (Gao et al., 2002).

2.9.3 Na/K-ATPase as a Signalling Molecule

In addition to the classical membrane transport function, Na/K-ATPase acts as signal transducing receptor in various cell types including cardiac myocytes, renal epithelial cells, neuronal cells and vascular smooth muscle cells and in sperm in presence of its specific ligand ouabain (Liu et al., 2000; Xie and Askari, 2002; Xie, 2003; Liu and Askari, 2006; Tian et al., 2006; Thundathil et al., 2006; Pierre et al., 2008; Tian and Xie, 2008; Zhang et al., 2008; Quintas et al., 2010; de Juan-sanz et al., 2013; Reinhard et al., 2013; Xie et al., 2015; Singh et al., 2015; Aperia et al., 2016; Peng et al., 2016; Cui and Xie., 2017; Madan et al., 2017). Two ouabain binding sites

have been reported on the α subunit which varies in affinity towards ouabain (Morrill et al., 2008; Sandtner et al., 2011; Khalid et al., 2014). The Na/K-ATPase is located in caveolae in the plasma membrane associated with its signalling partners such as Src-a non-receptor protein tyrosine kinase, which plays an important role in Na/K-ATPase induced signal transduction in porcine kidney cells (Wang et al., 2004; Liu and Askari 2006; Liu et al., 2011). The α subunit of Na/K-ATPase has two caveolin binding motifs (CBM), one in the cytosolic N-terminal domain near the first transmembrane domain (TM1) and the other in the TM10 on the extracellular side (Xie and Cai, 2003). These two CBMs on the α subunit are located away from its catalytic domains indicating the binding of Na/K-ATPase to caveolin does not affect the ion pumping function of Na/K-ATPase. However, the CBMs on the cytosolic N-terminal domain (TM1) are located close to the ouabain binding domain of the α subunit, supporting a signalling role, rather than enzymatic role, for Na/K-ATPase, because ouabain-induced interaction of Na/K-ATPase with caveolins is essential for signal transduction in porcine kidney cells (LLC-PK1 cells; Wang et al, 2004). Other than in the raft, Na/K-ATPase is also distributed in the non-raft platforms on the pig kidney cells cell membrane along with its signalling partners like Src, PI3K and EGFR (Liu et al., 2011).

2.9.4 Ouabain-Na/K-ATPase Mediated Signalling Pathway

Various signalling pathways reported to be induced by ouabain-Na/K-ATPase interaction includes MAPK pathway, cAMP-PKA pathway, PLC-PKC pathway and PI3K-Akt pathway which all activate multiple protein kinases and finally induce tyrosine phosphorylation of various proteins that elicit specific functions by target cells.

2.9.4.1 MAPK Pathway

MAPK pathways are cascades of important signal transducing enzymes present in mammalian cells connecting external stimuli to specific targets within the cell to regulate critical cellular process including cell survival, cell motility, cell growth, cell division, metabolism, migration, proliferation, differentiation, and death. The MAPK cascade is regulated by three groups of protein kinases that are activated in series: the MAPK Kinase Kinase or MEK kinase (MAPKKK or MEKK); MAPK kinase (MAPKK, MEK); and MAPK. Each of these activates specific protein kinases which further phosphorylate particular effector proteins to regulate various cell functions. Mainly three groups of MAPK families have been characterized in mammals: the ERK1/2; the C-Jun NH2-terminal kinases (JNK) 1/2/3; and p38 kinases. Each of these MAPKs are activated by specific MAPKKs: ERK 1/2 by MAPKK1/2; JNK1/2 by MAPKK 5/7; and p38 by MAPKK 3/6. Similarly, each of the MAPKKs are activated by specific MAPKKKs making this signalling more complex. Each MAPKKK gets stimulated by specific signals. For example, MAPKKK - rapidly accelerated fibrosarcoma (Raf) gets activated by growth factors which further activate ERK1/2 while stress stimuli activate other MAPKKKs and subsequently activate JNK or p38 MAPKs.

Membrane-associated non-receptor tyrosine kinase Src plays an intermediary role in many signal transduction pathways induced by Na/K-ATPase. Src is found to be attached to the α subunit of Na/K-ATPase through the SH2 and kinase domain and forms Src-Na/K-ATPase signalling complex in the cell membrane (Banerjee et al., 2015). In the Src-Na/K-ATPase signalling complex, Src remains in its inactive state, but binding of ouabain to Na/K-ATPase activates the Na/K-ATPase-bound Src. The activated Na/K-ATPase-Src complex then transactivates EGFR by Tyr-p of multiple residues on EGFR. Phosphorylated EGFR then phosphorylates the adaptor protein Shc,

which in turn activates the binding of the Grb2-Sos (Growth factor receptor-bound protein 2; Son of sevenless) complex. Activated Sos, which is a guanine nucleotide exchange factor, facilitates the activation of rat sarcoma protein (Ras) by exchanging guanosine diphosphate for guanosine triphosphate (GTP). Activated GTP-bound Ras then stimulates the classical MAPK cascade, which is a serine/threonine protein kinase cascade sequentially activating Raf (MAP3K), MEK (MAP2K), and ERK1/2 (Liu et al., 2007, Zhang et al., 2008, Reinhard et al., 2013).

The MAPK signalling pathway is also regulated by cross-communication with other signalling pathways. The cAMP-PKA signalling pathway regulates MAPK cascade depending on cell and receptor type. In fibroblasts cells, smooth muscle cells, and adipocytes, cAMP partially inhibits the MAPK pathway by inhibiting Raf-1 and thereby inhibits the growth of these cells (Stork and Schmitt, 2002). However, in PC12 cells cAMP induces activation of MAPK through PKA-induced activation of B-Raf (Yehia et al., 2001; Fey et al., 2012). PKC is another signalling molecule which regulates the MAPK pathway in various cell types. Along with Ras, PKC isoforms also have been shown to directly activate Raf-1 and subsequent MAP kinase activity in many cell types (PLC/DG/PKC/Raf/MEK/ERK1/2) (Schonwasser et al., 1998).

2.9.4.2 cAMP/PKA Pathway

In mammalian cells, stimulation of G-protein coupled receptors by specific agonist activates the G-proteins which then activate sAC. Activated sAC then catalyzes the synthesis of cAMP from ATP leading to an increase in the intracellular concentration of cAMP. The concentrations of cAMP in cells are regulated by the counterbalance between its synthesis by ACs and its degradation by the phosphodiesterases (PDEs). The cAMP then stimulates PKA which phosphorylates the serine/threonine residues of various target proteins leading to the activation of various protein kinases and/or inhibition of protein phosphatases, which finally activate various

tyrosine kinases leading to an increase in the phosphorylation of tyrosine residues of various proteins. Studies by Peng et al. (2016) reported that the ouabain-Na/K-ATPase interaction could promote the cAMP production by the activation of AC and subsequent activation of cAMP-dependent PKA signalling pathways in rabbit atrial cells. The cAMP-PKA path is one of the critical signalling pathways inducing sperm capacitation in various species. Various capacitation inducing agents like heparin have been reported to act by stimulating the cAMP-PKA pathway (Parrish, 2014). Ouabain has also been seen to induce cAMP-PKA mediated signalling to increase the Tyr-p and capacitation of bull sperm (Newton et al., 2010).

2.9.4.3 PLC/PKC Pathway

Activated Na/K-ATPase/Src/EGFR complex can also initiate PLC signalling cascades. An activated Src/EGFR complex activates PLC which further activates/catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP₂) to produce 1, 2-diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP₃). Both IP₃ and DAG act as second messengers. IP₃ binds to Ca²⁺ ion channels on endoplasmic reticulum (ER) and induce the release of Ca²⁺ from ER into the intracellular space in somatic cells. In spermatozoa which lack ER, Ca²⁺ is released potentially from the acrosome or mitochondria (Costello et al., 2009). DAG and Ca²⁺ act together to activate PKC (Zhaokan et al., 2005; Zhang et al., 2008; Reinhard et al., 2013; Aperia et al., 2016). PKC then activates target proteins to get the cellular response. Other than the PLC-mediated activation of inositol trisphosphate receptor (IP₃R), direct interactions of Na/K-ATPase and IP₃R also activate the Tyr-p and subsequent activation of IP₃R independent of activation by PLC (Miyakawa-Naito et al., 2003; Zhang et al., 2008).

2.9.4.4 PI3K/Akt Pathway

Ouabain bound Na/K-ATPase is also involved in the activation of phosphatidylinositol-3 kinase (PI3K) mediated signalling pathway in mammalian cells. PI3Ks are lipid kinases that phosphorylate the membrane lipid PIP2 at the 3-hydroxyl group to produce phosphatidylinositol 3,4,5-trisphosphate (PIP3). The increased PIP3 level then activates phosphoinositide-dependent kinase-1 (PDK1). The PDK1 interacts with downstream effector protein molecule Akt (also known as protein kinase B /PKB) to induce phosphorylation. Akt is a serine and threonine kinase which phosphorylates various effector molecules and subsequent signalling to induce cell-specific effects (Liu et al., 2007, Ye et al., 2013). The activation of Src receptor is important, but not necessary for the activation of PI3K/Akt (Wu et al., 2013).

2.9.5 Isoform Specificity of Na/K-ATPase α Subunit on Ouabain-Induced Signalling

Na/K-ATPase $\alpha 1$ is the subunit isoform which is abundant in most mammalian cells. Hence, most studies on ouabain-induced signalling in mammalian cells reflect signalling induced by the $\alpha 1$ isoform. Na/K-ATPase $\alpha 1$ is found closely associated with Src kinase, and on ouabain binding, Na/K-ATPase $\alpha 1$ induces signalling pathways including Src/EGFR/Ras/Raf/MEK/ERK1/2, PLC/PKC and PI3K/Akt pathways which are directly or indirectly mediated through Src.

Mammalian cells expressing exclusively $\alpha 2$ or $\alpha 3$ are unknown, and so much less information is available about the signalling properties of $\alpha 2$ or $\alpha 3$. Specific mammalian cell lines (Xie et al., 2015) expressing Na/K-ATPase $\alpha 2$ in the absence of any detectable amount of $\alpha 1$ polypeptide showed that ouabain-bound $\alpha 2$ did not stimulate Src kinase or downstream signalling molecules such as ERK and Akt but could still maintain the ion pumping action. The LM- $\alpha 3$ -1 mammalian cell line that expresses Na/K-ATPase $\alpha 3$ in the absence of any detectable $\alpha 1$ showed

that $\alpha 3$ similarly does not involve Src in signalling, instead $\alpha 3$ activates ERK through a Src-independent pathway involving PI3K and PKC. This also suggests that the activation of Src, while important, is not necessary for the activation of PLC/PKC, and PI3K/Akt pathways, i.e. ouabain binding to either $\alpha 2$ or $\alpha 3$ Na/K-ATPase isoforms does not activate Src-dependent signal transduction. Again, $\alpha 3$ is different from $\alpha 2$, in that $\alpha 3$ signals in a Src-independent manner whereas $\alpha 2$ does not induce any of the signalling activated by $\alpha 3$. In conclusion, signalling induced by the α subunit is distinct for each α isoform.

2.9.6 Na/K-ATPase in Sperm

Similar to somatic cells, mammalian spermatozoa contain Na/K-ATPase (Zhao and Buhr, 1996) which is involved in maintaining the ionic balance for normal functioning of the sperm (Jimenez et al., 2010; McDermott et al., 2015). Bull sperm express the ATPase subunit isoforms $\alpha 1$, $\alpha 3$, $\alpha 4$, $\beta 1$, $\beta 2$ and $\beta 3$ and are more concentrated on the HPM (Zhao and Buhr, 1996; Hickey and Buhr, 2012; Rajamanickam et al., 2017). Thundathil et al. (2006) reported a novel signalling pathway for bull sperm capacitation involving Na/K-ATPase as a signal transducer in the presence of ouabain, which has since been confirmed (Newton et al., 2010). Ouabain-Na/K-ATPase interaction induced multiple signalling pathways in bull sperm involving receptor tyrosine kinases (EGFR), non-receptor tyrosine kinases (Src), PKA, PKC and ERK1/2 to induce tyrosine phosphorylation and capacitation (Newton et al., 2010; Rajamanickam et al., 2017). Although the involvement of Na/K-ATPase as a signalling molecule in sperm capacitation has been confirmed, the specific subunit isoforms of Na/K-ATPase involved in capacitation are not yet clear. Since rafts are signalling platforms, ATPase isoforms that are concentrated in HPM raft domains would have a higher probability of involvement in capacitation-associated signalling. But the exact

membrane domain localization of various subunit isoforms of ATPase on sperm plasma membrane is not precisely known. In a recent study on bull sperm, Rajamanickam et al. (2017) identified $\alpha 1$ and $\alpha 4$ ATPase isoforms in both raft and non-raft fraction of whole sperm homogenates along with their signalling partners Src, EGFR and ERK1/2, as in renal cells (Liu et al., 2011). It was also reported that capacitation of bull sperm induced by ouabain increased the phosphorylation of these downstream signalling molecules in both the raft and non-raft fractions suggesting the presence of signalling pool of ATPase on the non-raft (Rajamanickam et al., 2017).

2.10 Progesterone

Progesterone is another endogenous steroid hormone that can induce capacitation and/or AR in at least some species of mammalian sperm. Structurally P4 (4-pregnen-3-20-dione) contains a 21-carbon steroid backbone called 4-pregnen with two ketone groups at the 3rd and 20th position. Progesterone is synthesized from its precursor pregnenolone, catalyzed by the enzyme 3 β -hydroxysteroid dehydrogenase. The main serum metabolites of P4 are 17 α -OH-progesterone (catalyzed by 17-hydroxylase), desoxycorticosterone (catalyzed by 21-hydroxylase), and pregnanediol (main urinary metabolite).

Classically, the actions of P4 are mediated by the intracellular progesterone receptor (PR), which when activated by the hormone modulates a specific set of gene expression in target cells (Tsai and O'Malley, 1994). The classical PR contains three conserved functional domains, including the N-terminus, a centrally located DNA binding domain, and C-terminal ligand binding domain (Graham and Clarke, 1997). Two PRs termed A (94 kDa), and B (120 kDa) are found in mammals. The amino acid sequences of both isoforms are the same, except that PR-B has 164 extra amino acids at the N-terminus of the protein, termed activation domain 3 (AF3) (Wen et al., 1994). Both isoforms have the same DNA binding domain and ligand binding domain. Although

they have the same capability to bind P4, PR-B is usually a stronger transcriptional activator than PR-A due to its AF3 domain.

In addition to the classical genomic action via nuclear PR isoforms, P4 can also act directly via non-classical membrane-bound receptor (mPR). A group of G protein-coupled cell surface receptors mediates a variety of rapid cell surface-initiated P4 actions in the reproductive system via modulation of intracellular signalling cascades and produce effects on the target tissue (Correia et al., 2007). The mPR is totally different from the genomic PR in size, but the P4 binding region is preserved. Three different isoforms of mPR have been demonstrated in human cells namely mPR- α , mPR- β , and mPR- γ (Correia et al., 2007). The P4-binding sites in sperm mPR share homology with the steroid binding site of the genomic PR; however, they don't share homology to either the DNA-binding domain or amino-terminal domains of the genomic PR (Luconi et al., 1998) and these mPRs in sperm are not affected by genomic PR antagonists, such as RU486 (Emiliozi et al., 1996). The non-genomic membrane receptors on sperm show a lower affinity for P4 compared to the genomic PR in the cytosol of somatic cells (Flesch and Gadella, 2000). In human sperm, two surface receptors with different affinity for P4 (one in the nanomolar and the other in the micromolar range) have been identified (Luconi et al., 1998). However, the exact nature of mPR or the difference in the affinity of each isoform to P4 is not yet clear.

2.10.1 P4 Actions on Sperm for Regulating Capacitation and AR

Generally, capacitation begins following the removal of seminal plasma from the sperm surface in the female reproductive tract which is believed to be stimulated by components in the oviductal fluid like P4 (Baldi et al., 2009). In spermatozoa, the P4 effects are mediated via non-genomic mPR located on the plasma membrane which stimulates intracellular signalling to

regulate various functions including capacitation, hyperactivation, and AR in various species (Luconi et al., 2002; Therian and Manjunath, 2003; Baldi et al., 2009). Various molecules are described as mPRs on sperm plasma membrane in different species. This includes a 52 kDa protein on the mouse sperm head membrane (Thomas et al., 2005), two mPR with a molecular weight 54 and 57 kDa on human sperm (Luconi et al., 1998) and a 71 kDa protein on boar spermatozoa (Jang and Yi, 2005). These mPRs are found to be localized in the acrosomal region of sperm (Wu et al., 2005), but the specific molecule which acts as mPR on mammalian sperm and the subsequent signalling pathway involved, is not yet clear.

The P4 acts through mPRs, stimulating intracellular signalling pathways including Ca^{2+} -induced PLC-DAG/IP3-PKC, and MAPK pathway leading to Tyr-p of sperm (Luconi et al., 1998; Thundathil et al., 2002; Ickowicz et al., 2012; Jin and Yang, 2016). However, the response of sperm to P4 is highly variable depending upon species. Progesterone induces capacitation or the acrosomal reaction in equine and human sperm (Witte and Schafer-Somi, 2007; McPartlin et al., 2008; Sagare-Patil et al., 2013), but responses of bovine sperm to P4 are variable, with P4 either only inducing capacitation, or only stimulating the AR in previously-capacitated sperm or inducing both capacitation and acrosomal reaction (Therien and Manjunath, 2003; Lukoseviciute et al., 2004; 2005). The variable responses may be due to the concentration of P4 or the physiological state of sperm, composition of the capacitating medium, or other unknown factors.

The availability of the mPRs on the sperm surface increases after capacitation because of the removal of surface coating components during capacitation which is proposed to uncover PRs on the sperm plasma membrane (Emiliozi et al., 1996). So, after the release of the sperm from the oviductal epithelium following capacitation, the sensitivity of sperm to P4 increases in the oviduct. High concentrations of P4 rapidly increase intracellular Ca^{2+} in both capacitated and non-

capacitated sperm of several mammalian species (Harper et al., 2006). But the exact mechanism of P4 dependent Ca^{2+} increase is still under investigation. Possibly P4 directly activates Ca^{2+} channels like CatSper or indirectly via voltage-dependent Ca^{2+} channels (Gonzalez-Martinez et al., 2002; Mannowetz et al., 2017). Progesterone primed calcium influx makes the spermatozoa more susceptible to hyperactivation and AR (Sumigama et al., 2015).

2.10.2 Na/K-ATPase as a P4 Receptor

P4 can act as a signal transducer in amphibian oocytes, acting through membrane-bound Na/K-ATPase (Morrill et al., 2005; Morrill et al., 2008). In these oocytes, P4 binds with the low-affinity ouabain binding site on Na/K-ATPase, which as mentioned above is structurally similar to steroid receptors and can accommodate steroids like P4 with planar configuration (Seccombe et al., 1989). Both P4 and ouabain compete for this low-affinity ouabain binding site on the α subunit of ATPase in the amphibian oocyte (Morrill et al., 2005). But P4, which can efficiently displace ouabain from canine kidney Na/K-ATPase (Seccombe et al., 1989), has a slightly higher affinity than ouabain for the low-affinity site on amphibian oocyte (Morrill et al., 2008). Binding of P4 to this low-affinity site stimulates a conformational change permitting caveolin to bind to the Na/K-ATPase, and the caveolin-ATPase complex then stimulates various signalling molecules including the diacyl glycerol (DAG) and the MAP kinase signalling cascade (Duckworth et al., 2002; Morrill et al., 2012). This is highly reminiscent of the intracellular Tyr-p activated by ouabain in cardiac myocytes and in bull sperm for the induction of capacitation (Wang et al., 2004; Thundathil et al., 2006; Newton et al., 2010).

Since Na/K-ATPase-ouabain interaction is confirmed to be important for inducing bull sperm capacitation, better understanding about the actual role of Na/K-ATPase and the molecular

mechanisms associated with it for inducing capacitation would provide insights for developing methodology for predicting bull sperm fertility at younger reproductive stages.

2.11 Research Hypotheses

1. Although the involvement of Na/K-ATPase in bull sperm capacitation has been confirmed, the exact subunit isoforms involved in capacitation-associated signaling are not known. Since the raft signaling platforms are involved in capacitation events, it is possible that the Na/K-ATPase subunit isoforms which are involved in signaling preferentially localize in rafts on the sperm HPM.

H₀: Specific isoforms of Na/K-ATPase are differentially located in raft and non-raft areas of bovine sperm HPM.

2. Both ouabain and P4 have been identified as independent physiological inducers of capacitation. As both ouabain and P4 can act with the same binding site on Na/K-ATPase in amphibian oocyte membranes, there is a very exciting possibility that ouabain and P4 may interact at Na/K-ATPase in mammalian sperm plasma membrane to regulate induction of capacitation.

H₀: Ouabain and P4 interact in regulation and induction of bovine sperm capacitation.

3. Fertility-related differences in the function of various Na/K-ATPase isoforms have not been studied in bull sperm. It is possible that both ouabain and P4 differentially induce bull sperm capacitation in sperm from bulls of varying fertility.

H₀: Ouabain and P4 act differently in inducing capacitation in bull sperm of different fertilizing ability.

2.12 Objectives of the Study

1. Isolation of rafts from bovine sperm HPM.
2. Identification and comparison of the isoforms of α and β subunits of Na/K-ATPase in the rafts and non-raft domains of sperm HPM.
3. Elucidation of the interaction (synergism/competition) of progesterone and ouabain on inducing bull sperm capacitation.
4. Illumination of the fertility-related difference in the action of ouabain and P4 on inducing bull sperm capacitation.

3.0 LOCALIZATION OF NA/K-ATPase ISOFORMS IN BULL SPERM HEAD PLASMA MEMBRANE RAFTS

3.1 Introduction

After ejaculation, mammalian spermatozoa must undergo biochemical and physiological modifications in the female reproductive tract to acquire the ability to fertilize an oocyte. These functional changes are termed capacitation (Chang, 1951; Austin, 1952). Capacitation causes the removal of decapacitating factors from the sperm surface leading to the exposure of sperm surface molecules, which are responsible for the interaction of spermatozoa with the oocyte.

Capacitation is initiated by signals that the sperm receives in the female reproductive tract. These signalling events start at the sperm head plasma membrane (HPM) where sperm first interact with the egg. Capacitation is associated with the remodelling of the sperm plasma membrane and beginning of specific internal changes which are necessary for successful fertilization. One of the major changes seen in sperm plasma membrane during capacitation is the cholesterol efflux induced by albumin and other high-density lipoproteins present in the female reproductive tract (Davis, 1981; Visconti et al., 2002). Cholesterol efflux causes reorganization of membrane lipids and phospholipids, modifies the activity of associated transmembrane proteins and increases the fluidity of the plasma membrane (Gadella et al., 2008; Nicolson, 2014). These functional membrane modifications occur mainly in the sperm HPM and are important for sperm-zona pellucida binding, acrosome reaction (AR) and membrane fusion during fertilization (Caballero et al., 2012). Tyrosine phosphorylation (Tyr-p) of sperm proteins is another critical molecular response associated with capacitation (mouse: Visconti et al., 1995; cattle: Thundathil et al., 2006; pigs: Awda et al., 2009 and human: Battistone et al., 2014). Various signalling pathways reported to be activated during mammalian sperm capacitation include cAMP/PKA pathway, PLC/PKC

pathway (Ickowicz et al, 2012; Rotfeld et al, 2014), PI3K/Akt pathway (Aquila et al., 2004) and ERK 1/2 pathway (Awda and Buhr 2010; Rajamanickam et al., 2017).

Thundathil et al (2006), reported a novel signalling pathway for bull sperm capacitation involving Na/K-ATPase as a signal transducer in the presence of ouabain, which has since been confirmed (Newton et al., 2010). Ouabain is an endogenous cardiac glycoside that acts as an endogenous steroid hormone in mammals (Schoner and Scheiner-Bobis, 2007). Endogenous ouabain is found in blood plasma and is also present in nanomolar concentrations in bovine semen and vaginal fluids (Daniel et al., 2010). Na/K-ATPase is a heterodimeric, amphipathic membrane protein present in many cell types. Na/K-ATPase consists of two main polypeptide subunits, alpha (α) with four known isoforms: $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$, and beta (β) subunit with three isoforms: $\beta 1$, $\beta 2$ and $\beta 3$ (Geering, 2008). Functional Na/K-ATPase exists as a dimer ($\alpha\beta$) or double dimer of $[(\alpha\beta)_2]$ of α and β subunits (Linnertz et al., 1998). Na/K-ATPase is concentrated in the HPM (Zhao and Buhr, 1996) and isoforms $\alpha 1$, $\alpha 3$, $\beta 1$, $\beta 2$, and $\beta 3$ have been confirmed and quantified in bull sperm HPM (Hickey and Buhr, 2012). The α subunit contains the binding site for ouabain, which stimulates the signalling task of Na/K-ATPase (Kaplan, 2002). The binding of α with β subunit is critical for ouabain receptivity and enzyme action of Na/K ATPase. The existence of four α and three β isoforms suggests the possibility of occurrence of different combinations of these subunits forming different isozymes of Na/K-ATPase.

In somatic cells including cardiac cells, signalling platforms involve specific membrane rafts which regulate various cellular functions. Rafts are discrete membrane domains formed by the aggregation of different lipids mainly sphingomyelins and cholesterol which are packed together along with various signalling proteins and glycosylated phosphatidyl inositol (GPI) anchored proteins to form a liquid ordered microdomain (Simone and Toomre, 2000; Simons and

Sampaio, 2011). Rafts house various signalling molecules including Src family kinases, G proteins, MAPK, PKC, PLC, PI3K and some enzymes like RTK including EGFR and PDGF (Simons and Toomre, 2000; Xie, 2003; Chini and Parenti, 2004; Calder and Yaqoob, 2007; Jahn et al., 2009). Rafts are heterogeneous (Simons and Sampaio, 2011), but certain molecules like GPI-linked protein CD59, the ganglioside GM1, and other proteins like flotillin-1, flotillin-2 and caveolin-1 are enriched in all kinds of rafts and have been used as raft markers (Cross, 2004). Co-localization of several signalling components into a raft enhances molecular interactions making signalling more efficient (Pike, 2006). Due to their structural characteristics, rafts are insoluble in non-ionic detergents like Triton X-100 at low temperatures and can be isolated as DRMs by density gradient floatation of homogenized cellular materials (Brown and Rose, 1992; Kawano et al., 2011).

Rafts have been identified and isolated from the spermatozoa of various mammalian species (mouse, guinea pig: Travis et al., 2001; human: Cross, 2004; boar: (Khalil et al., 2006); bull: (Post et al., 2010); ram: Colas et al., 2012). In most of the experiments rafts were isolated from the whole sperm. Rafts are involved in mammalian sperm capacitation (Khalil et al., 2006). Since the events associated with capacitation begin in the HPM of spermatozoa, the presence of raft in the sperm HPM would support its involvement in capacitation. During capacitation, rafts cluster at the apical HPM, and these reorganizations are associated with the redistribution, localization and association of different membrane proteins in zona recognition and AR (Mayorga et al., 2007; Gadella et al., 2008; Brewis and Gadella, 2009; Tapia et al., 2012; Boerke et al., 2014). Although the involvement of Na/K-ATPase as a signalling molecule in sperm capacitation has been confirmed, the specific subunit isoforms of Na/K-ATPase involved in capacitation are not yet clear. Since rafts are signaling platforms, ATPase isoforms that are concentrated in HPM raft domains would have a higher probability of involvement in capacitation-associated signaling.

Therefore, we hypothesized that rafts exist in the HPM of bull sperm, and that some ATPase isoforms would preferentially locate in HPM rafts.

3.2 Materials and Methods

3.2.1 Chemicals and antibodies

The following reagents were acquired from Thermo-Fisher Scientific (Unionville, ON, Canada): albumin standards, dextrose, dimethyl sulfoxide, disodium hydrogen phosphate anhydrous (Na_2HPO_4), disodium hydrogen phosphate monohydrate ($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$), glycine, hydrogen peroxide 30 %, methanol HPLC grade, poly ethylene glycol (PEG 8,000), sodium azide, sodium dodecyl sulfate (SDS), sucrose and tween 20. The following reagents were purchased from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada); 30 % acrylamide/bis solution 29:1, N, N, N', N'-tetramethylethylene-diamine (TEMED), ammonium persulfate, precision plus protein Western C standards (detect bands ranging from 10-250 kDa), protein assay dye reagent concentrate, triton X-100 and precision plus strep- tacin- horse radish peroxidase (HRP) conjugate. Complete mini protease inhibitor mini cocktail tablets (EDTA free) were from Roche Diagnostics (IN, USA), percoll from GE Healthcare (Mississauga, ON, Canada), and live: dead sperm viability kit from Molecular Probes Inc. (Eugene, OR). Immobiline-P PVDF transfer membrane (pore size 0.45um) was purchased from Millipore Canada Ltd, (Etobicoke, Canada). The bromophenol blue, dextran, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), luminol, magnesium chloride ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$), N- α -p-tosyl-L-lysinechloromethyl ketone (TLCK), p-coumaric acid, pepstatin-A, phenylmethanesulfonyl fluoride (PMSF), potassium chloride (KCl), sodium chloride (NaCl), tris-HCl, , trizma base,

trizma hydrochloride, Carestream® Kodak® autoradiography GBX developer/replenisher, Carestream® Kodak® autoradiography GBX fixer and replenisher, Carestream® Kodak® X-Omat LS film were purchased from Sigma-Aldrich, Canada Ltd (Oakville, ON, Canada). For protein identification, A431 cell lysate (positive control for $\beta 3$), anti-Na/K-ATPase $\beta 3$ (mouse monoclonal; Clone 46), anti-caveolin-1 (mouse monoclonal; Clone 2297), anti-flotillin-1 (mouse monoclonal; Clone 18), anti-flotillin-2 (mouse monoclonal; Clone 29), human endothelial cell lysate (positive control for flotillin-2 and caveolin-1) and rat cerebrum lysate (positive control for flotillin-1) were obtained from BD Bio-sciences (Mississauga, ON, Canada), anti- Na/K-ATPase $\beta 2$ (mouse monoclonal IgG2a; ab76509) was from Abcam Inc. (Cambridge, USA), anti- Na/K-ATPase $\alpha 3$ (mouse monoclonal IgG1; clone XVIF-G10) and HeLa cell lysate (positive control for $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$ and $\beta 2$) were purchased from Enzo Life Science (Farmingdale, NY, USA), anti-Na/K-ATPase $\beta 1$ (mouse monoclonal IgG2AK; clone C464.8), anti-Na/K-ATPase $\alpha 1$ (mouse monoclonal; IgG1K; clone C464.6), anti-Na/K-ATPase $\alpha 2$ (rabbit monoclonal IgG2AK; clone C464.8), goat anti-mouse IgG-HRP conjugate (polyclonal) and goat anti-rabbit IgG-HRP conjugate (polyclonal) were purchased from EMD Millipore Corporation (Single Oak Drive, Temecula, USA).

3.2.2 Semen Collection and Evaluation

All procedures met the requirements of the University of Saskatchewan ethics and animal care requirements (Animal Use Protocol number: 20140082). Ejaculates were collected from mature Holstein bulls ($n = 20$) using live teasers at Semex (Guelph, ON, Canada) and volume, motility and concentration determined as for commercial processing. Ejaculates with an average motility of $85 \pm 8.15 \%$ were then diluted with egg yolk free ‘Clear extender’ (proprietary

composition, Semex) to a concentration of 60 Million/ml and transported at 18°C to the laboratory within 24 hours. The extender is designed to slow down the progressive motility of the sperm to allow it to be viable for up to 1 week at 18°C. The extended ejaculates after reaching the laboratory were warmed to room temperature (45-60 min), assessed for motility and a small aliquot of this ejaculate was diluted to 1×10^7 spermatozoa per ml of phosphate buffered saline (PBS: 125mM NaCl, 8mM Na₂HPO₄, 2mM NaH₂PO₄.H₂O, 5mM KCl and 5mM dextrose; pH adjusted to 7.4) for assessing the viability of the sperm. Motility was assessed with a Hamilton-Thorne motility analyzer (Version 14 HTM-IVOS) using a prewarmed 20 µm deep Leja standard count four chamber slide (Leja products B.V., Luzernestraat 10, 2153 GN Nieuw-Vennep, The Netherlands) at 37°C. The analytical setup of motility analyzer was: frame rate, 60 Hz; frames acquired, 10 Hz; minimum contrast, 80 pixels; minimum cell size, 5 pixels; threshold straightness, 70 %; medium average path velocity (VAP) cutoff, 50 microns/sec; low VAP cutoff, 30 microns/sec; static size limits, 0.49–4.86 pixels; static intensity limits, 0.6-2.16 pixels; static elongation limits, 8 % – 68 %; nonmotile head size, 10 pixels; and nonmotile head intensity, 101. Viability of the diluted sperm were assessed with Sybr-14/Propidium Iodide (PI) staining (Awda et al, 2009), counting 2×100 spermatozoa as live (green) or dead (red) cells using a fluorescent microscope (Laborlux S; Leitz, Germany) fitted with a blue filter (450–490 nm) under 40x objective. Ejaculates were discarded if they had fewer than 50 % viable sperm on arrival at the laboratory.

3.2.3 HPM Isolation

All steps were performed at room temperature unless specified otherwise. Ejaculates from 2 bulls were pooled for isolating sufficient quantity of HPM (n=16). After warming the pooled ejaculate, the extender was removed by centrifugation (800x g, 10 min) followed by washing the

sperm pellets again in PBS (800x g, 10 min). The final pellet was diluted with PBS [1:4 volume:volume (v:v)] and used for further processing. The HPM was isolated as before (Hickey and Buhr, 2012). Briefly, the diluted semen was washed with 35 % percoll (1:2 semen:percoll, v/v) and the percoll removed from the pellet by dilution in PBS and centrifugation (800 x g; 10 min; thrice). The final pellets were resuspended in PBS and subjected to nitrogen cavitation (Parr Cell Disruption unit, 650 psi, 10 min). The extruded solution (known as the cavitate) was centrifuged (800 x g; 10 min), the supernatant was collected and was layered onto phase partition tubes (3.94 g 20 % dextran, 1.97 g 40 % PEG, 0.19 g PBS, 2.42 g 1.5 M sucrose). These tubes were mixed, centrifuged (800 x g; 10 min, twice), and harvested the top portions. The top layer was washed with PBS (206,000 x g, 20 min, 5°C; twice). The final pellet, which was the HPM, was harvested with TNE buffer (10 mM Tris- HCl, 0.15 M NaCl, 5mM EDTA) the protein concentration was measured using the Bio-Rad protein assay and aliquoted in to two tubes. One aliquot of the purified HPM was snap-frozen in liquid nitrogen and stored (-80°C) for western blot analysis within three days, the remainder was used for raft isolation.

3.2.4 Isolation of Raft and Non-Raft Fractions

Raft and non-raft fractions were obtained from the HPM by the method of Khalil et al, (2006). A total of 20 different bulls provided ejaculates for the 16 HPM/raft preparations; each preparation contained semen from a different pair of bulls, although two bulls each provided three ejaculates and eight bulls each provided two. The purified HPM was lysed by gentle stirring for 1 hr in ice cold lysis buffer (TNE, 1 % vol:vol Triton X-100, 0.2 mM TLCK (N- α -p-tosyl-L-lysine chloromethyl ketone); total buffer volume had 3:1 TritonX: HPM wt:wt). The lysate was then mixed with equal volume of 85 % (w/v) sucrose in TNE to a final sucrose concentration of 42.5

% (wt/v) sucrose, and 1 ml was transferred into an ultracentrifuge tube (Polypropylene Quick-seal tubes, Beckman Coulter) and overlaid with 3 ml of 30 % and 1.75 ml 5 % sucrose in TNE for centrifugation (200000 g, 18 hr, 4°C,) in a swinging bucket rotor (SW 41 Ti, Beckman Coulter). After centrifugation, 1 ml fractions were collected from top to bottom and the pellet at the bottom was discarded. These isolated 1 ml fractions were assessed for protein concentration (Bio-Rad protein assay) as well as their absorbance at A₄₀₀ using a spectrophotometer. Fraction with high light scattering intensity was considered as the raft fraction; all other fractions were pooled together and considered as the non-raft fraction. Both raft and non-raft fractions were concentrated separately using Amicon® Ultra centrifugal filters (3800 g, 4°C, 2 hr for non-raft & 8-10 hr for raft; the concentrated non-raft fraction was kept at 4°C until raft concentration was completed). The concentrated fractions were snap-frozen in liquid nitrogen and stored at -80°C for use on the next day for western blot analysis.

3.2.5 Western Blotting

To select the ideal antibody concentration and protein load which provide optimum visible band intensity for Na/K-ATPase isoforms, modifications were made from previously published research work (Hickey and Buhr, 2012). A preliminary study also confirmed the presence of different subunit isoforms of Na/K-ATPase in bull sperm HPM by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described by Hickey and Buhr (2012). Equal amounts of raft, non-raft and HPM fractions were subjected to SDS-PAGE and immunoblotting for the presence of the raft marker proteins caveolin-1, flotillin-1 and flotillin-2 (Cross, 2004). The $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$ and $\beta 3$ Na/K-ATPase isoforms in matched raft, non-raft

and HPM were also identified by SDS-PAGE and immunoblotting (n = 7 for α 1, β 3 & β 1, n = 6 for β 2, n = 5 for α 2 & α 3) using specific monoclonal antibodies.

Proteins in each fraction (raft, non-raft and HPM) were solubilized by boiling in sample buffer, as well as an aliquot from a matched pool of aliquots of bovine kidney membrane fraction as an internal inter-gel control (Hickey and Buhr, 2012). Electrophoresis (100V, 1.15 h) was performed using 10 % polyacrylamide running gels for detecting flotillin-1, flotillin-2 and for both α and β isoforms of Na/K-ATPase whereas 12 % gel was used for detecting caveolin-1. In addition to the bovine kidney, manufacturers' positive controls for each of the various proteins (A431 cell lysate for β 3; human endothelial cell lysate for flotillin-2 and caveolin-1; rat cerebrum lysate for flotillin-1 and HeLa cell lysate for α 1, α 2, α 3, β 1 and β 2) were run on the appropriate gels. After electrophoresis, the protein bands were electrotransferred to Immobiline-P PVDF membranes that were immunoblotted overnight (4°C) with the appropriate antibodies in TTBS: anti flotillin-1 (1:250), anti flotillin-2 (1:5000), anti caveolin-1 (1:100), and Na/K-ATPase α 1 (1:2500), α 2 (1:15000), α 3 (1:3500), β 1(1: 1000), β 2 (1:1000), and β 3 (1:250). After washing, all membranes were incubated for 1hr at room temperature with goat, anti-mouse IgG HRP conjugate (1: 2,500 in TTBS) except for α 2, which was incubated with goat, anti-rabbit IgG HRP conjugate (1:15000 in TTBS). Protein bands were detected with chemiluminescence (incubating PVDF membrane with 68 mM p-coumaric acid, 1.25 mM luminol and 3 % hydrogen peroxide for 2 minutes) and images were developed using Carestream® Kodak® X-OMAT LS Film (Carestream Health, Inc., Rochester, NY, USA) according to manufacturer's description. The sensitivity of the primary antibody for each isoform was tested by inclusion of the manufacturer's positive control in every gel, and also by incubating the membranes with secondary antibody in absence of primary antibody. The use of strep-tactin greatly improved the clarity of the Precision Plus Protein™ standards, but produced three bands (125, 81 and 71 kDa) in the bovine kidney inter-gel standard

that were absent when no strep-tactin was added. These strep-tactin-dependent bands were therefore considered artifacts and excluded from all further analyses.

The positive bands were analyzed using Image Quant TL 8.1 Software (GE Healthcare) for area (number of pixels in a given selection), volume (sum of the pixel intensity for all pixels in a given selection), and molecular weight (MW). Amount of each MW band (vol/mg protein loaded onto the gel) was corrected for gel variation using the inter-gel controls. The total amount of each isoform in each fraction was calculated by summing the corrected band volume per mg protein. The percentage of each band within an isoform for each of the raft, non-raft and HPM fractions was calculated as a measure of the relative dominance of each band (we called it enrichment) in each fraction by dividing the volume of each band over total volume of that isoform within the fraction.

3.2.6 Immunofluorescence

Localization of Na/K-ATPase $\alpha 2$ isoform on the HPM of bull sperm was confirmed by immunofluorescence assay ($n = 3$) as described previously by Hickey and Buhr (2012). Of the replicate samples used, two were pooled fresh ejaculates (extended) from 2 bulls each and the third was frozen semen. All steps were conducted at room temperature unless otherwise specified. For fresh extended ejaculate, initially the extender was removed by centrifugation (800x g, 10 min) followed by washing the sperm pellets in PBS by centrifugation (500x g, 5 min). The spermatozoa in the frozen straw washed with PBS by centrifugation (500x g, 5 min). The spermatozoa in the final sperm pellet were fixed by 1 % formaldehyde (vol:vol in PBS). The sperm were attached to poly-L-lysine-coated slides (1 mg/ml poly-L-lysine in PBS) by settling for 60 min at 4°C. Spermatozoa on the slides after washing (2×5 minutes in PBS) were either allowed to remain

intact or permeabilized with methanol (-20°C) for 30 seconds and washed again (2 x 5 minutes in PBS). Cells were then blocked by incubating in PBS containing 10 % normal goat serum (PBS+10 % NGS) for 30 min at 37°C followed by incubation with anti-Na/K-ATPase $\alpha 2$ primary antibodies (1:100 in PBS + 10 % NGS) for 60 min at 37°C. After washing the slides (2 x 5 min in PBS + 10 % NGS), spermatozoa were incubated with FITC-conjugated goat anti-rabbit IgG (1:50 in PBS +10 % NGS) for 60 min at 37°C followed by a final wash (2 x 5 min in PBS +10 % NGS). For control, instead of primary antibody, spermatozoa were incubated in PBS +10 % NGS followed by goat anti- rabbit IgG. A drop of antifade (0.1 % P-phenylene di- amine and 90 % glycerol in PBS) was placed over the spermatozoa, coverslips were mounted over it and cells were immediately observed under fluorescent microscope at FITC wavelengths (495 - 519 nm; Laborlux S; Leitz, Germany).

3.2.7 Mass Spectrometry

Mass spectrometry for detecting the $\alpha 2$ isoform in isolated HPM samples was done at Canadian Centre for Health and Safety in Agriculture (CCHSA) mass spectrometry laboratory, which is a part of the Core Mass Spectrometry Facility at the University of Saskatchewan. After denaturing the HPM proteins with trifluoroethanol, the proteins were digested with trypsin and the generated peptides were analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Zhang et al, 2011). The MS/MS spectra were acquired with a 6550 iFunnel quadrupole time-of-flight mass spectrometer (Agilent Technologies, Mississauga, ON, Canada) and processed with Agilent MassHunter BioConfirm (B.06.00). Resulting data was searched against the sequence databases Swiss prot (contain bovine fasta sequence) and a custom data base containing all the ATPase isoforms across many species that was prepared by gathering sequences from NCBI non-

redundant protein databases (NCBIInr) to identify proteins. Peptide identification was determined using a 0.3 Da fragment mass error and a parent mass error of 10 ppm assuming full tryptic specificity with up to one missed cleavage. The false discovery rate (FDR) threshold was set to 5 % for the protein matches.

3.2.8 Statistical Analysis

Since the band volume and band percentage data had a lognormal distribution, statistical analysis was conducted on the logarithmic transformed data using a Mixed model procedure (SAS; version 9.4; SAS Institute, Inc., Cary, NC). The band volume or band percentage of each protein were compared between different fractions (HPM, raft and non-raft) using preplanned contrasts. Comparing the amount of any one isoform across fractions is valid as we are using same antibody at identical concentration for detecting bands in each fraction. Significance and trends are reported at $P \leq 0.05$ and $0.05 < P < 0.10$, respectively.

3.3 Results

Even after 24 hours of shipping, the extended ejaculates had an average motility of 80 ± 7.55 % on arrival. After washing to remove extender and even after storage for 3 hours at 37°C the individual ejaculates used in this study had 73 ± 11.6 % viable and 63 ± 9.0 % motile sperm (mean \pm SE). Preliminary experiments ($n = 5$) with HPM from extended ejaculate detected all ATPase isoforms reported by Hickey and Buhr (2012), displaying similar MW bands as that from fresh ejaculate.

3.3.1 Optical Density and Protein Distribution in the Sucrose Density Gradient

Overall, protein concentration in the sucrose density gradient used for raft isolation increased from the less-dense to more-dense fractions (Fig. 3.1 A). The fourth fraction had the highest light scattering intensity ($P < 0.05$; Fig. 3.1 B). Frequently there was a pellet at the bottom of the tube below fraction 6; this was discarded. The raft and non-raft fractions contained 2.9 % and 35.9 %, respectively, of the total HPM protein initially loaded on the gradient.

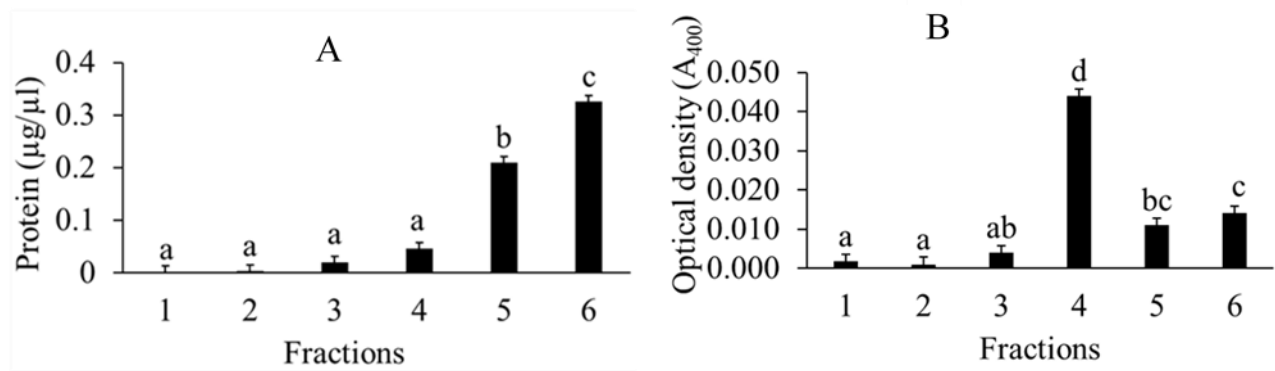


Figure 3.1: (A) Protein concentration (µg/µl) and (B) optical density (OD; at A₄₀₀) in fractions obtained from sucrose density gradients of head plasma membrane-Triton X-100 lysate (n = 18). An analysis of variance was performed on the data and actual means are displayed. a-d means without a common letter differ between fractions ($P < 0.05$).

3.3.2 Western Blotting and Mass Spectrometry

The sensitivity of the primary antibody for each isoform was confirmed by detecting positive signal at the described molecular weight in the manufacturer's positive control in every gel, and the complete absence of positive signal in membranes incubated with secondary antibody without primary antibody. The amount of protein loaded into the wells of SDS gels for ATPase

immunoblots were confirmed by alpha-tubulin density. Alpha-tubulin was not used for protein density of raft immunoblots, as tubulin is differentially distributed between raft and non-raft fractions (Wolff, 2009).

Immunoblots of the HPM and raft, but not the non-raft, fractions were positive for the specific raft marker flotillin-1 (45686 ± 25957 ; 48747 ± 9903 ; 0 ± 0 vol in pixel/mg protein in HPM, raft and non-raft respectively; $n = 7$; Fig. 3.2). The raft marker proteins caveolin-1 & flotillin-2 were not detectable in HPM, raft or non-raft fractions ($n = 2$).

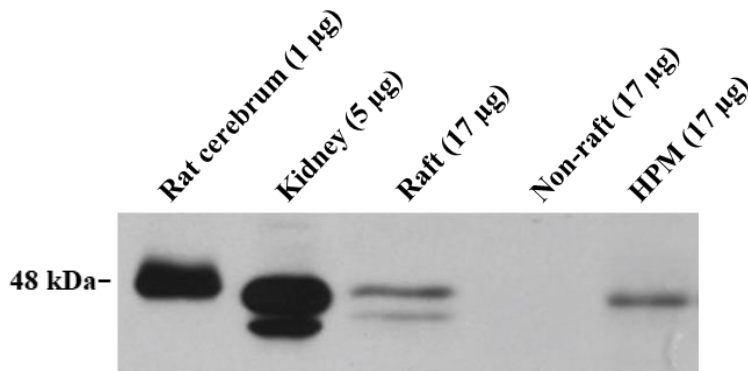


Figure 3.2: Western immunoblot of flotillin-1 in the raft, non-raft and head plasma membrane fractions of bovine sperm; Amount of protein (μg) used to run the gel for each sample is indicated in brackets. Rat cerebrum lysate was the manufacturer's positive control and bovine kidney microsomal membranes acted as an inter-gel standard. HPM = head plasma membrane.

The isoform $\alpha 2$ was detected in bull sperm HPM for the first time. The WB detected seven $\alpha 2$ bands in the HPM (MW range 129 ± 2.3 to 25 ± 0.7 kDa, mean \pm SE; Fig. 3.3), of which only three appeared in the raft fraction (129, 54 and 25 kDa). Each of these three $\alpha 2$ bands was significantly enriched in the raft fraction, being much more concentrated there than in the HPM (for 129 kDa, 15 ± 5.6 % of all raft $\alpha 2 > 5 \pm 0.9$ % of HPM $\alpha 2$; for 54kDa, 36 ± 13.2 % of raft $\alpha 2 > 7 \pm 2.4$ % of HPM; for 25kDa, 63 ± 18.3 % raft $> 3 \pm 0.9$ % HPM; all $P < 0.05$, Appendix 1.1).

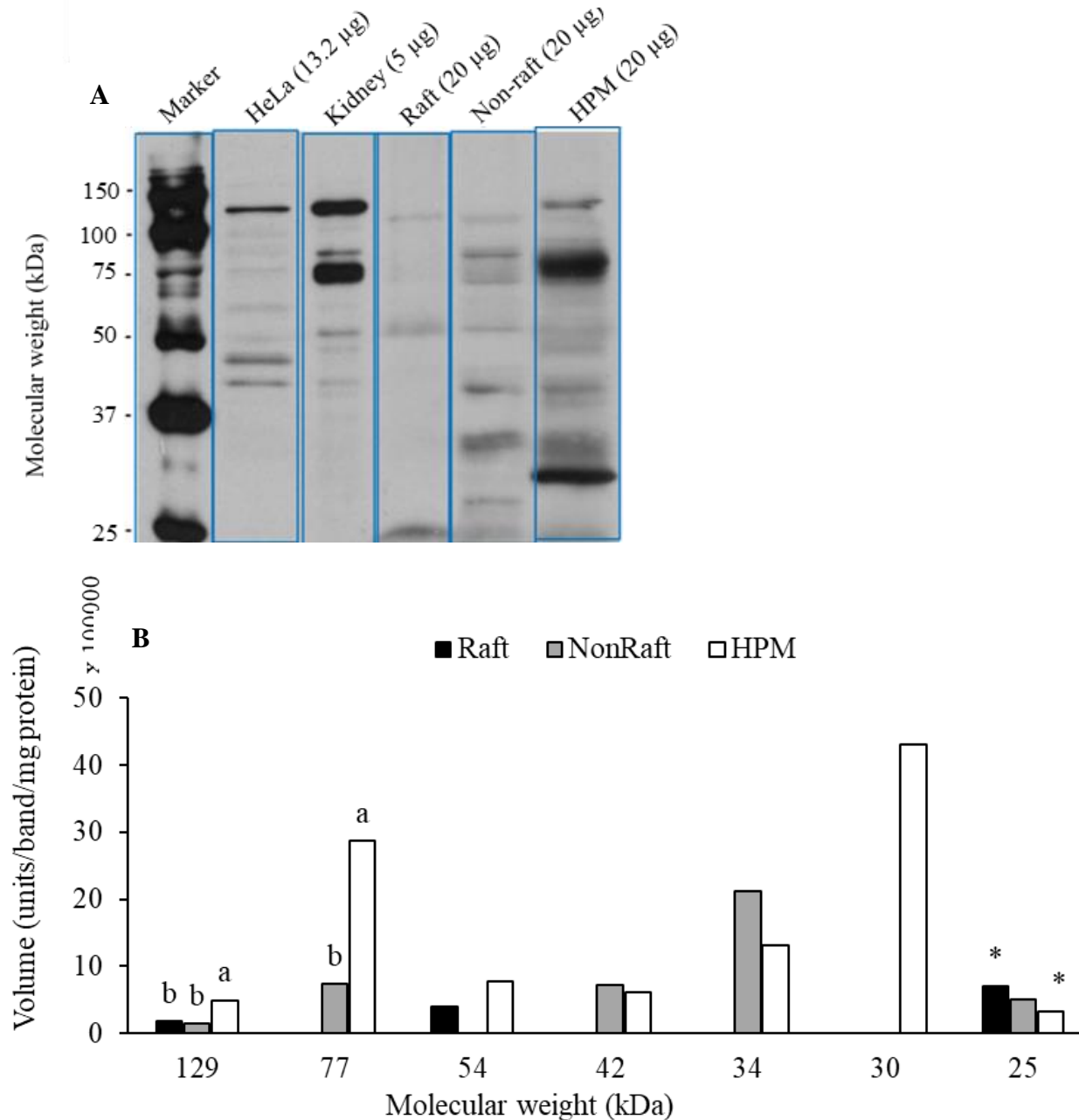


Figure 3.3: Na/K-ATPase α 2 in the bovine sperm head plasma membrane and its raft and non-raft fractions (A) Typical Western immunoblot of Na/K-ATPase α 2 in the bovine sperm head plasma membrane (HPM), raft and non-raft fractions of the HPM, HeLa cell lysate and bovine kidney microsomal preparation. Amount of protein (μ g) used to run the gel for each sample is indicated in brackets. (B) Amount of Na/K-ATPase α 2 in the HPM, raft and non-raft fractions (Mean; $n = 5$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$). * values tend to differ $P = 0.06$.

The presence of Na/K-ATPase $\alpha 2$ on bull sperm was also confirmed by immunofluorescent labelling with specific monoclonal antibody against the $\alpha 2$ isoform which detected some level of fluorescence on the sperm head both on the intact and permeabilized cells while the sperm tail did not show any fluorescence (Fig.3.4; A and B). Incubation of sperm with secondary antibody alone (control) elicited no fluorescence on both the intact and permeabilized cells (Fig.3.4; C and D) confirming the specificity of the antibodies. To ensure that all sperm were visualized and to observe its morphology, white field images of all fluorescent fields were analyzed. Closer examination of the sperm head found that on intact cells the $\alpha 2$ antibody showed uneven patches of fluorescence on the acrosome as well as on the equatorial segment while the permeabilized cells showed more intense patchy fluorescence mainly on the apical acrosome (Fig.3.4; A and B).

The $\alpha 2$ detected in western blot and immunofluorescent labelling was also identified by LC-MS/MS. Aligning the data to both Swiss Prot and the custom data base using sequences from NCBI nr identified multiple peptides of $\alpha 2$ (at 113.50 kDa) with a percentage coverage (percentage of peptides identified compared to total sequence of the entire protein) of 8.6 and 42.1 respectively for the databases. The Swiss Prot data base detected six peptide sequences ranging in length from 6-13 amino acids and the NCBI nr identified five of those same sequence and four additional sequences as characteristics of $\alpha 2$.

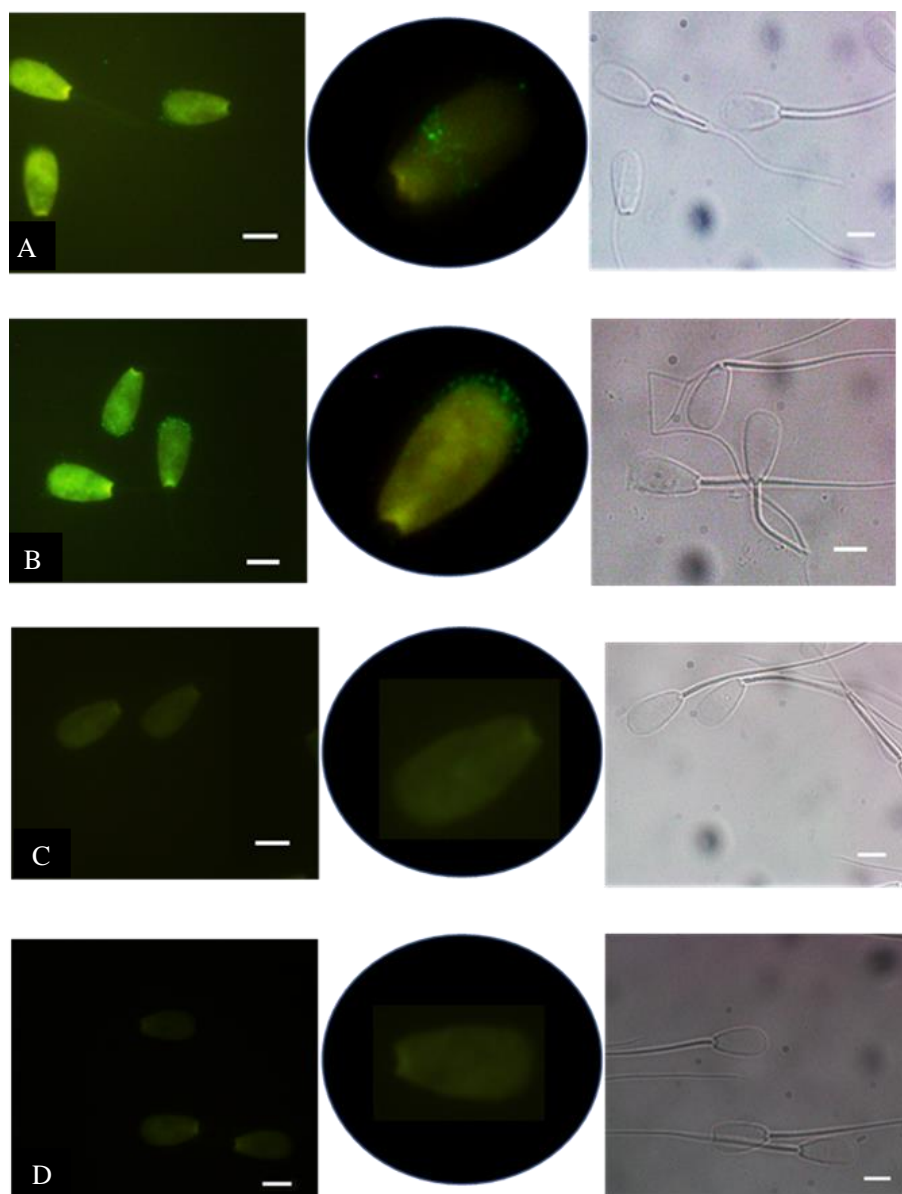
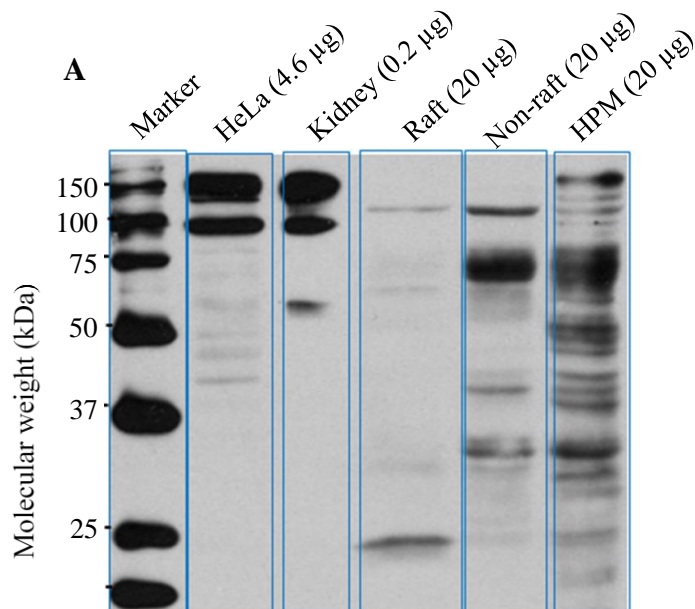


Figure 3.4: Immunolabelling of Na/K-ATPase $\alpha 2$ in bovine sperm. Typical immunofluorescent and their bright field images of (A) intact and (B) methanol-permeabilized bull sperm using Na/K-ATPase $\alpha 2$ specific primary and FITC-conjugated secondary antibody. Control sperm (C: Intact and D: Permeabilized) incubated in PBS +10% NGS and FITC-conjugated secondary antibody did not show any fluorescence. Center column of oval images are enlarged sperm head showing the typical fluorescent pattern. Scale bar represent 5 micrometers.

Western blotting detected 16 $\alpha 1$ bands in HPM (MW range 158 ± 2.0 to 22 ± 0.5 kDa, mean \pm SE), with raft fraction having only five of these and non-raft fraction eight (Fig. 3.5). Three of these five raft bands were significantly enriched in the raft fraction compared to the HPM (for 110kDa, 22 ± 7.1 % raft $\alpha 1 > 4 \pm 1.1$ % of HPM; for 72 kDa 28 ± 13.2 % raft $\alpha 1 > 17 \pm 4.6$ % HPM; for 25kDa, 61 ± 21.5 % of raft $\alpha 1 > 11 \pm 3.6$ % of HPM: all $P < 0.05$; Appendix 1.2). The bands at 110 ± 2.4 , 72 ± 1.5 , 64 ± 1.4 , 31 ± 0.6 and 25 ± 0.7 kDa were present both in the raft and non-raft fractions of HPM whereas the bands at 40 ± 0.8 , 39 ± 0.7 and 34 ± 0.6 were present only on the non-raft fractions of HPM. Among the bands which are present both in the raft and non-raft bands, the 25 ± 0.7 kDa band was enriched in the raft fraction compared to non-raft fraction (61 ± 21.5 % of raft $\alpha 1 > 2.4 \pm 1.1$ % of non-raft) while the other bands contributed equally in both the fractions (Appendix 1.2).



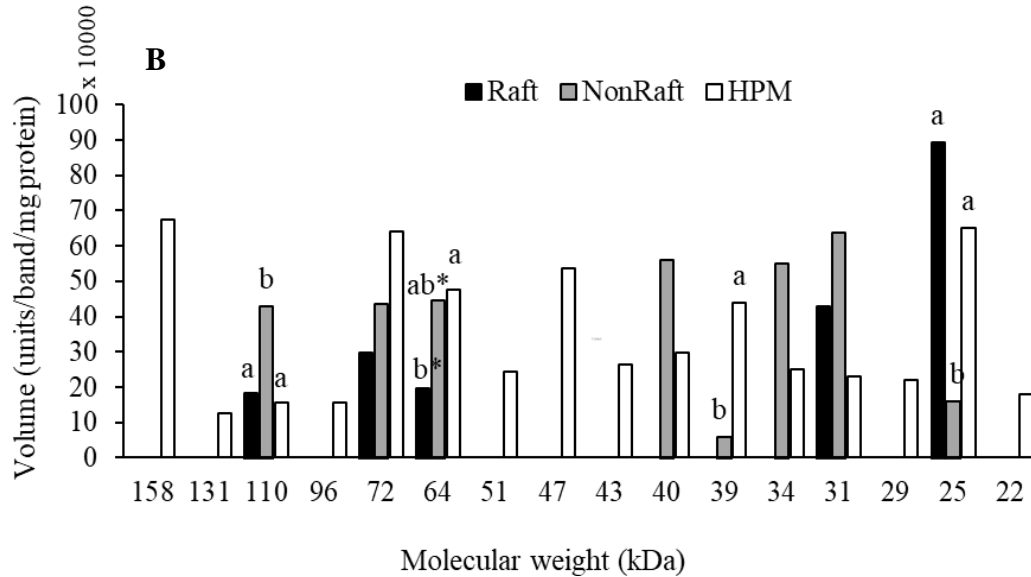


Figure 3.5: Na/K-ATPase $\alpha 1$ in the bovine sperm head plasma membrane and its raft and non-raft fractions (A) Typical western immunoblot of Na/K-ATPase $\alpha 1$ in the head plasma membrane (HPM), raft and non-raft fractions of the HPM, HeLa cell lysate and bovine kidney microsomal preparation. Amount of protein (μg) used to run the gel for each sample is indicated in brackets. (B) Amount of Na/K-ATPase $\alpha 1$ in the HPM, raft and non-raft fractions (Mean; $n = 7$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$). * the two values tend to differ $P = 0.07$.

Of the 14 $\alpha 3$ bands present in the HPM (MW range 154 ± 4.0 to 21 ± 0.6 kDa, mean \pm SE), raft fraction had four and the non-raft fraction five (Fig. 3.6). Both raft and non-raft fractions had bands at 114, 68, 55 and 25 kDa while only non-raft fraction had band at 40 kDa. The 25 kDa band was significantly enriched both in the raft and non-raft fraction compared to the HPM (84 ± 19.3 % of raft $\alpha 3 = 64 \pm 19.3$ % of non-raft $\alpha 3 > 5 \pm 1.1$ % of HPM $\alpha 3$, $P < 0.05$; Appendix 1.3); all other bands contributed equally to their membrane.

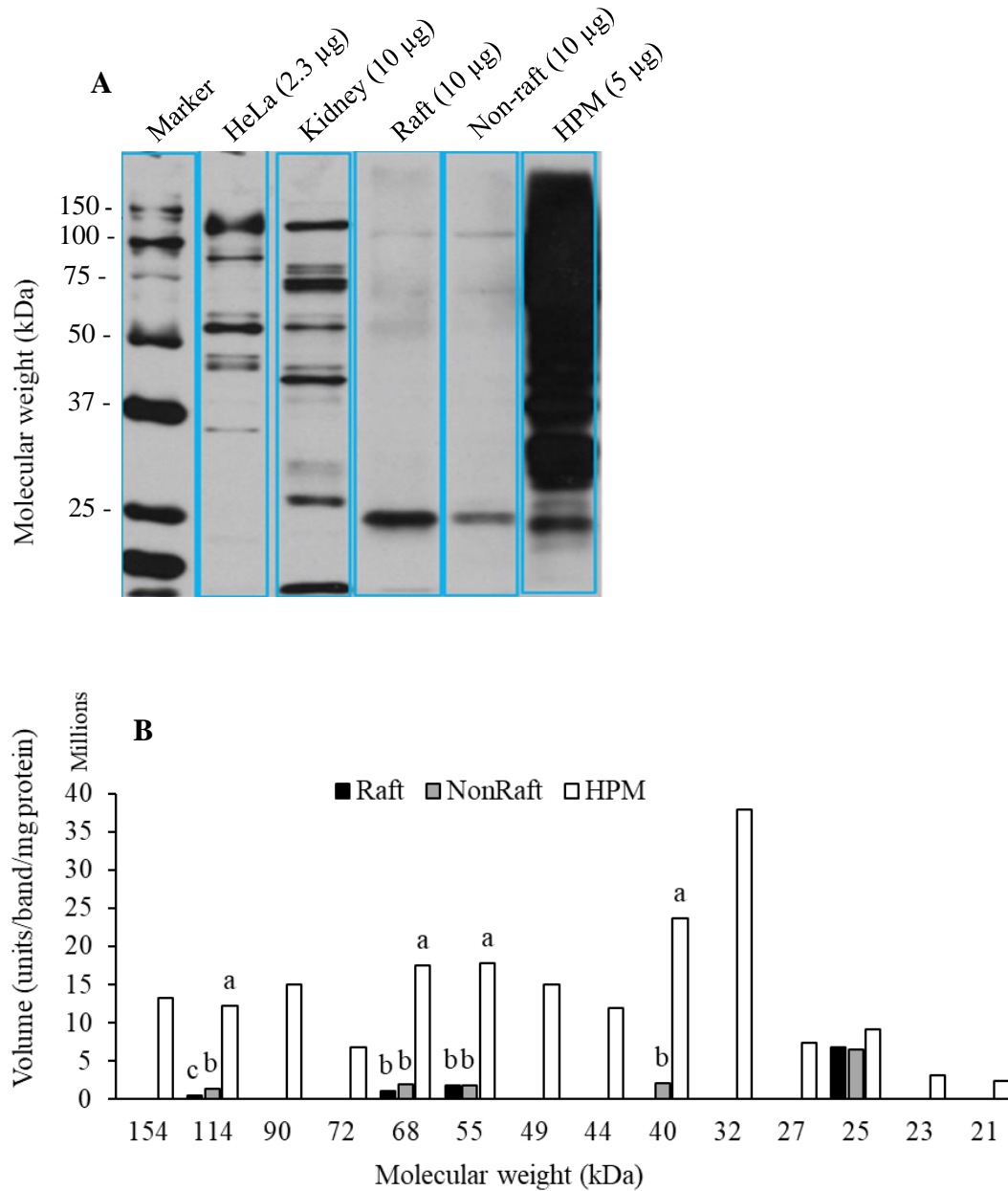
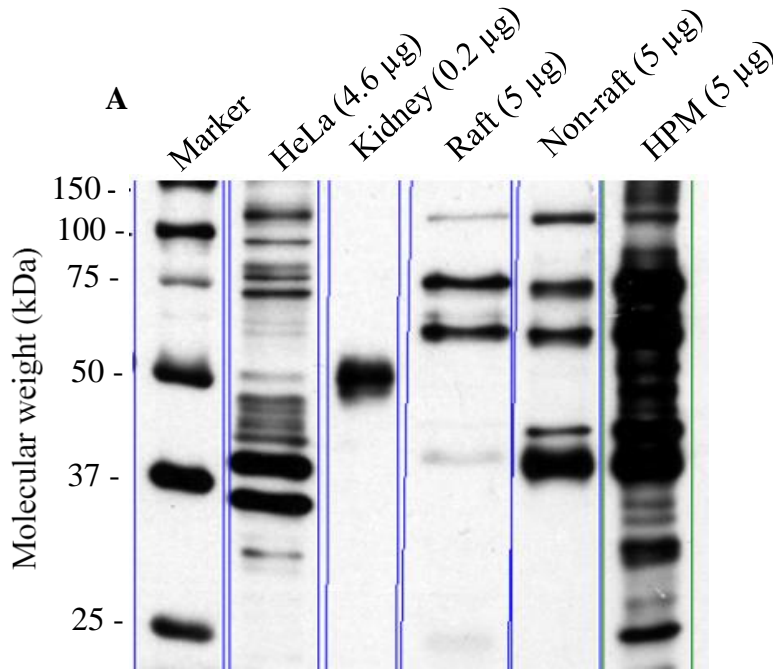


Figure 3.6: Na/K-ATPase $\alpha 3$ in the bovine sperm head plasma membrane and its raft and non-raft fractions (A) Typical western immunoblot of Na/K-ATPase $\alpha 3$ in the bovine sperm head plasma membrane (HPM), raft and non-raft fractions of the HPM, HeLa cell lysate and bovine kidney microsomal preparation. Amount of protein (μ g) used to run the gel for each sample is indicated in brackets (B) Amount of Na/K-ATPase $\alpha 3$ in the HPM, raft and non-raft fractions (Mean; $n = 5$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$).

The raft fraction showed eight of the 11 β 1 bands present in the HPM (MW range 103 ± 1.84 to 17 ± 0.33 kDa; Fig. 3.7) while the non-raft fraction showed only six of the HPM bands. The raft fraction had significantly less of the 50 and 24 kDa bands than HPM ($P < 0.05$). The 56 kDa band was more prominent in the raft fraction than in the HPM ($60 \pm 14.7 \% > 21 \pm 6.7 \%$; raft $>$ HPM, $P < 0.05$; Appendix 1.4), while other bands at 71, 50, 42, 39 and 24 kDa were present in equal proportions in both raft fraction and HPM. The 50 and 24 kDa HPM bands were detected only on the raft fraction of HPM and was absent on the non-raft fraction. Six bands at molecular weights 103, 71, 56, 42, 39, and 17 kDa were present both in the raft and non-raft fractions and had similar volume bands except at 39 kDa band where the raft fraction had significantly lower volume than the non-raft fraction.



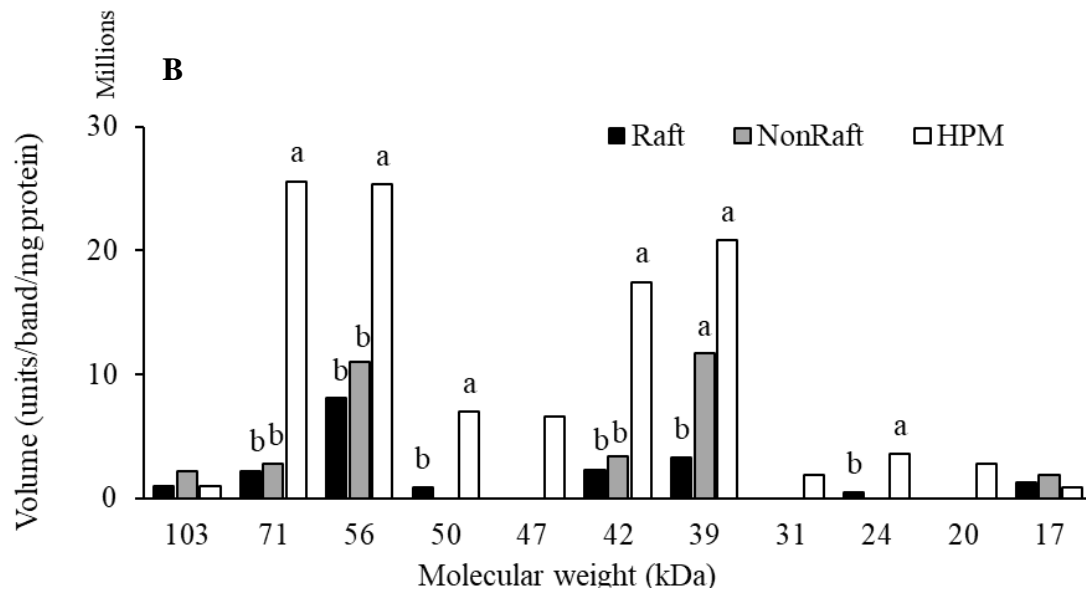


Figure 3.7: Na/K-ATPase β 1 in the bovine sperm head plasma membrane and its raft and non-raft fractions (A). Typical western immunoblot of Na/K-ATPase β 1 in the head plasma membrane (HPM), raft and non-raft fractions of the HPM, HeLa cell lysate and bovine kidney microsomal preparation. Amount of protein (μ g) used to run the gel for each sample is indicated in brackets (B) Amount of Na/K-ATPase β 1 in the HPM, raft and non-raft fractions (Mean; $n = 7$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$).

There were 10 β 2 bands present in the HPM (MW range 112 ± 1.1 to 22 ± 1.1 kDa) with both raft and non-raft fractions had four of those bands (Fig. 3.8). The 25 kDa HPM bands were detected only on the raft fraction and HPM but was highly concentrated and enriched in the raft fraction compared to the HPM ($60 \pm 13.6\% > 3 \pm 0.8\%$; respectively, of all β 2 present, raft > HPM, $P < 0.05$; Appendix 1.5). The 40 kDa band was specifically concentrated on the non-raft fraction but had significantly less volume than in the HPM ($P < 0.05$). Three bands at 112, 73 and 33 kDa were present both in the raft and non-raft fractions of HPM, of which the 112 and 73 kDa bands had higher volume in the non-raft than in the raft fraction ($P < 0.05$), while the 33 kDa bands had similar volume in both fractions.

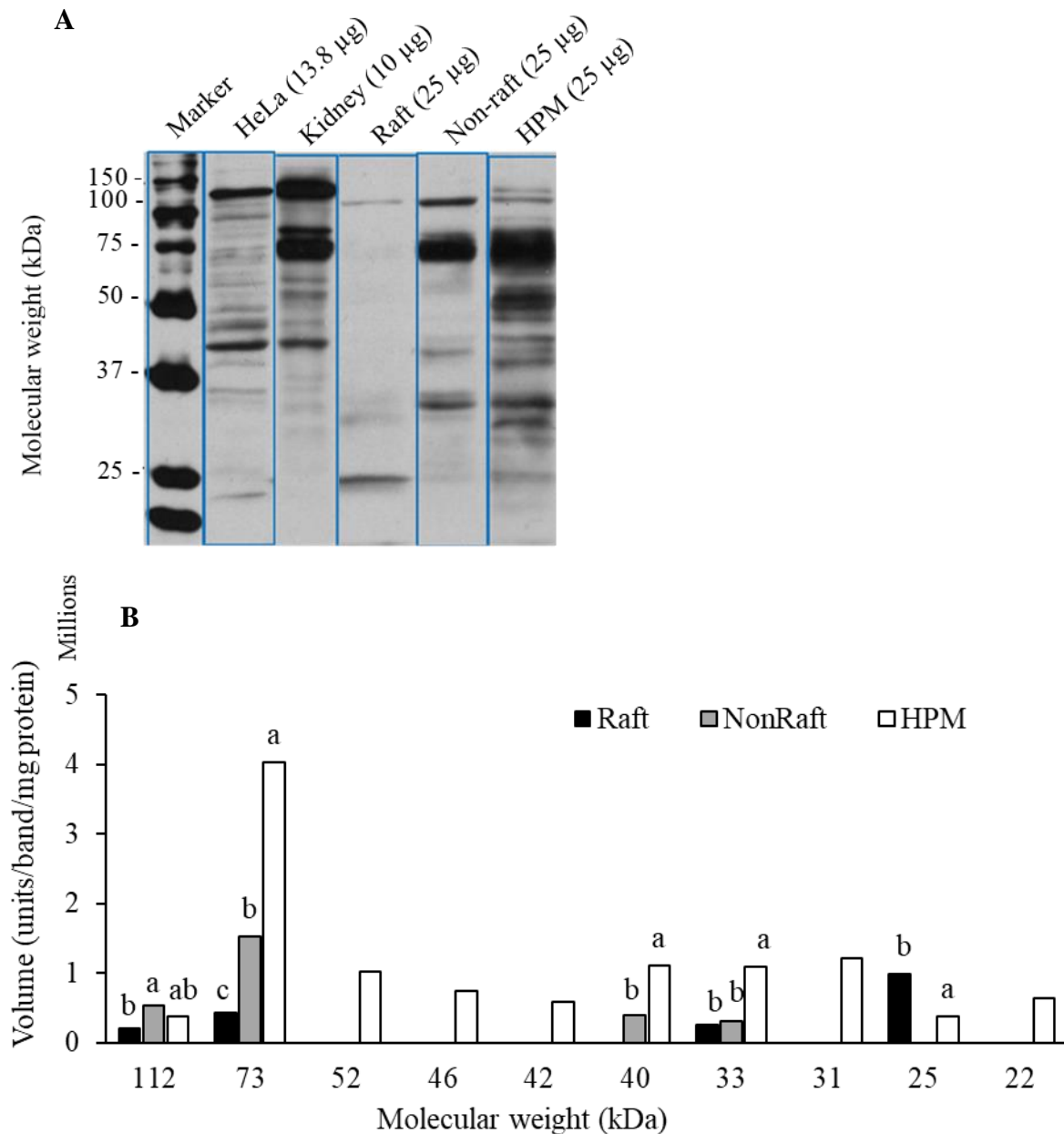
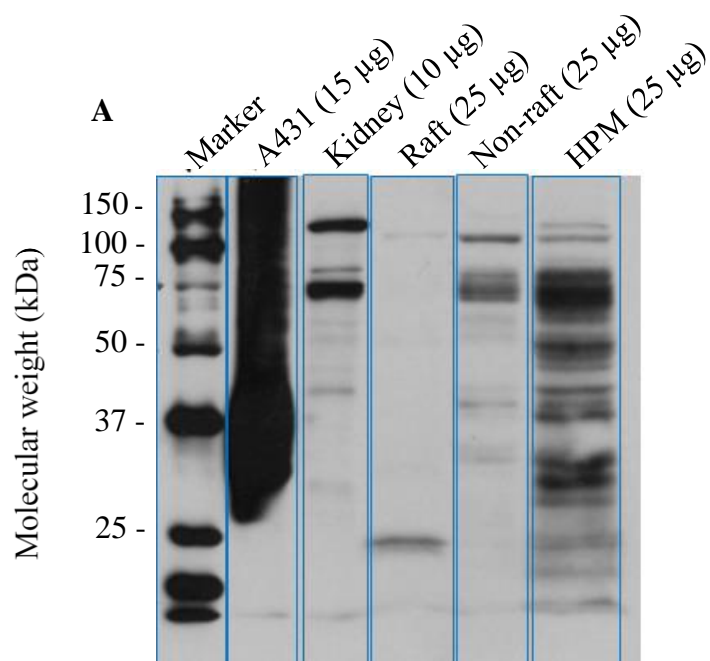


Figure 3.8: Na/K-ATPase β 2 in the bovine sperm head plasma membrane and its raft and non-raft fractions (A) Typical western immunoblot of Na/K-ATPase β 2 in the bovine head plasma membrane (HPM), raft and non-raft fractions of the HPM, HeLa cell lysate and bovine kidney microsomal preparation. Amount of protein (μ g) used to run the gel for each sample is indicated in brackets (B) Amount of Na/K-ATPase β 2 in the HPM, raft and non-raft fractions (Mean; $n = 6$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$).

Of the 13 $\beta 3$ bands present in the HPM (MW range 114 ± 0.9 to 17 ± 0.2 kDa), the raft fraction had six and non-raft fraction had eight bands (Fig. 3.9). The 31 kDa band was unique to the HPM and raft fraction but made up a smaller percentage of all the $\beta 3$ present in the raft fraction than HPM ($9 \pm 2.3 \% > 3 \pm 1.2 \%$; HPM $>$ raft, $P < 0.05$; Appendix 1.6). The 25 kDa band was highly significantly enriched in the raft fraction compared to the HPM ($71 \pm 18.6 \%$ of raft $\beta 3 > 6 \pm 1.6 \%$ of HPM; $P < 0.05$, Appendix 1.6) as was the more minor 114 kDa band ($8 \pm 2.3 \%$ of raft $\beta 3 > 3 \pm 0.7 \%$ of HPM $\beta 3$, $P < 0.05$, Appendix 1.6). In contrast, the 73 kDa bands was less prominent in the raft fraction than in the HPM ($3 \pm 1.1 \%$ of raft $\beta 3 < 24 \pm 6.8 \%$ of HPM, $P < 0.05$, Appendix 1.6).



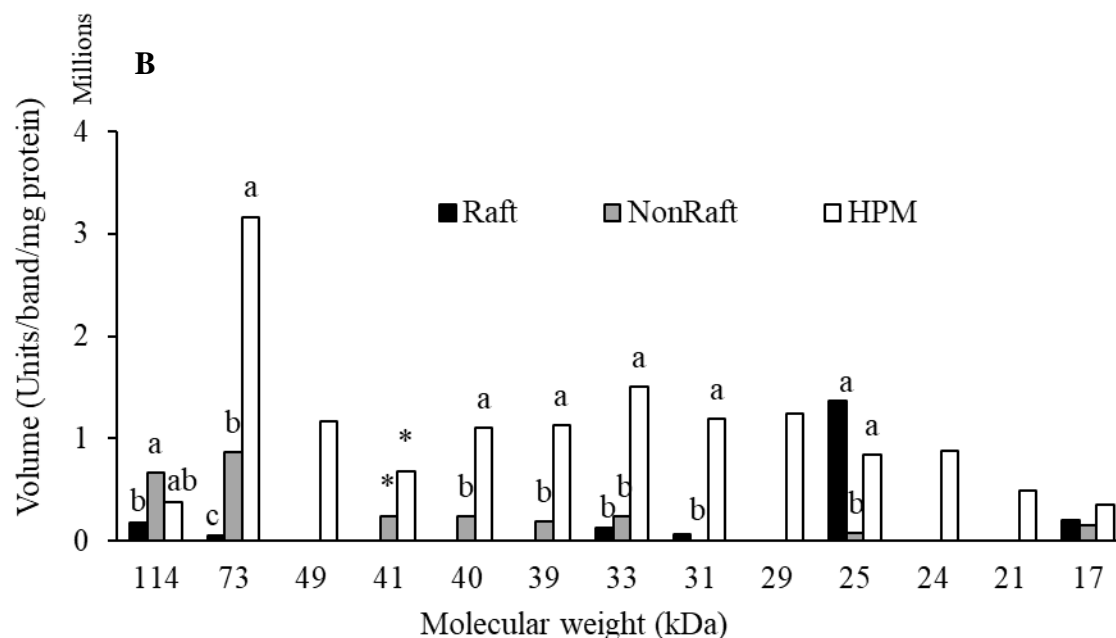


Figure 3.9: Na/K-ATPase β 3 in the bovine sperm head plasma membrane and its raft and non-raft fractions (A) Typical western immunoblot of Na/K-ATPase β 3 in the head plasma membrane (HPM), raft and non-raft fractions of the HPM, A431 cell lysate and bovine kidney microsomal preparation. Amount of protein (μ g) used to run the gel for each sample is indicated in brackets (B) Amount of Na/K-ATPase β 3 in the HPM, raft and non-raft fractions (Mean; $n = 7$). The volume of each band obtained from image quant analysis was corrected for protein load and gel to gel variation. Analysis was done on log-transformed normalized data and the means were compared between different fractions in each band using preplanned contrasts. Actual means are displayed in the figure. a-c means within a band without a common letter differ among fractions ($P < 0.05$). Bars in each band without superscripts did not differ between fractions ($P > 0.05$). * the two values tend to differ $P = 0.06$.

To know how much of each isoform in the HPM was actually located on the raft and non-raft fractions, we calculated the total amount of each isoform in the isolated HPM, raft and non-raft fractions (total vol/ μ g protein load from IQ for each fraction \times total protein in each fraction; Fig. 3.10, Appendix 1.7). Considering the total amount of ATPase from all isoforms, raft fraction had significantly less total Na/K-ATPase than in the HPM ($P < 0.05$). Within the raft fraction, there was significantly more $\beta 1$ and $\alpha 3$ than the other isoforms ($\beta 1=44 \% > \alpha 3=24 \% > \beta 2=11 \% > \beta 3=9 \% > \alpha 2=7 \% > \alpha 1=5 \%$ of total Na/K-ATPase in raft). HPM similarly had more $\alpha 3$ and $\beta 1$ than the other ATPase isoforms ($\alpha 3=48 \% > \beta 1=32 \% > \beta 2/ \beta 3/ \alpha 2=6 \% > \alpha 1= 2\%$ of total ATPase in HPM).

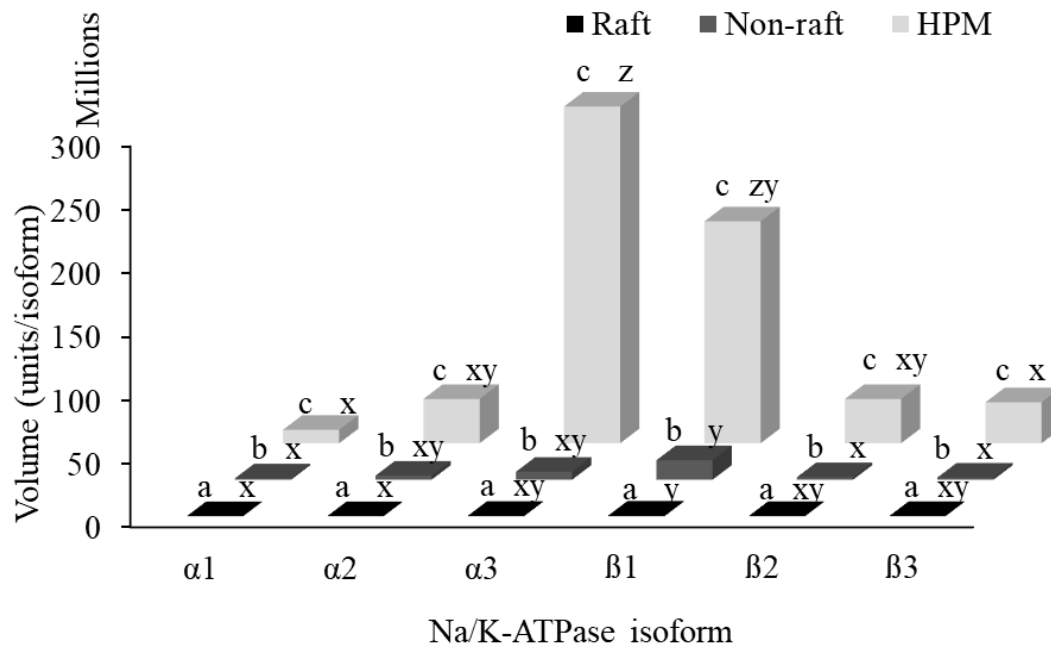


Figure 3.10: Total volume of Na/K-ATPase isoforms in the bovine sperm head plasma membrane and its raft and non-raft fractions (soluble) fractions (mean). Total volume of Na/K-ATPase was calculated by multiplying total volume/ μ g protein load from IQ for each fraction \times total protein in each fraction ($n = 7$). Analysis was done on log-transformed normalized data and the means were compared between different fractions in each isoform and between isoforms in each fraction using preplanned contrasts. Actual means are displayed in the figure. HPM = head plasma membrane

a-c means without a common letter differ among fractions within an isoform ($P < 0.05$)

x-z means without a common letter differ among isoforms within a fraction ($P < 0.05$)

3.4 Discussion

Results here conclusively show for the first time that rafts exist in the plasma membranes of the head of bovine sperm. Furthermore, the rafts contain the various α and β isoforms of Na/K-ATPase. This includes the $\alpha 2$ isoform, which has not previously been demonstrated in bovine sperm HPM.

When the trans-membrane receptor Na/K-ATPase is activated by the steroid hormone ouabain, it induces protein tyr-p and capacitation in bull sperm. Rafts, liquid ordered domains in the plasma membrane, frequently serve as platforms for signalling molecules initiating intracellular tyr-p and other signalling pathways. The existence of rafts in sperm HPM and having Na/K-ATPase isoforms present in rafts, suggests that this signalling could occur through these membrane platforms.

Rafts were separated as the opalescent layer on the low-density fractions of the density gradient, which had higher optical density (light scattering as a measure of solubilization) than the other fractions, consistent with other raft isolations (Khalil et al, 2006). Raft identity was also confirmed by using western blotting to seek marker proteins uniquely characteristic of rafts in other cell membranes. Flotillin-1, a known raft marker protein on bovine (Post et al, 2010), murine (Miranda et al, 2009) and porcine sperm (Van Gestel et al, 2005; Boerke et al, 2014) was present in the HPM and raft fractions, but not non-raft (Fig. 3.2). Flotillin-1 is primarily concentrated in the equatorial and post-acrosomal regions of the bovine sperm head (Post et al, 2010) and so would be expected to be detected in the HPM. Other raft marker proteins flotillin-2 and Caveolin-1 could not be detected in the raft fraction or HPM even though Caveolin-1 has been reported in whole bovine sperm (Post et al, 2010; Rajamanickam et al, 2017). Caveolin-1 is limited to the post-equatorial region of bovine sperm heads and prominent in the midpiece of bull sperm (Post et al.,

2010), explaining its detection in rafts isolated from whole sperm and its exclusion from our highly purified HPM (Zhao and Buhr, 1996). The combined physical characteristics of detergent insolubility, low density and the exclusive presence of the marker protein flotillin-1 all support that this fraction was indeed rafts native to the HPM. Previous studies have isolated rafts from porcine and bovine whole sperm and the involvement of raft on capacitation events on these species has been confirmed (Khalil et al, 2006; Rajamanickam et al, 2017)

To focus on signaling initiating capacitation, rafts were isolated from the purified HPM, not whole sperm as have been used in other studies (Khalil et al, 2006; Rajamanickam et al, 2017). Whole sperm could yield rafts from membranes of the principal piece, mitochondria, nucleus, and acrosome. Then to ensure the information was verifiable and reliable, immunoblots were prepared from HPM of multiple ejaculates, and all evident bands in all isoforms were identified by western immunoblotting and quantified. Preliminary studies on HPM from these extended ejaculates detected similar banding pattern for HPM isoforms of Na/K-ATPase as previously seen from fresh ejaculates (Hickey and Buhr, 2012) confirming that components in the extender did not change the Na/K-ATPase profile in bull sperm HPM. Each isoform's bands were confirmed to align with the manufacturer's own antibody control. Knowing that any of the identified bands could reflect modifications such as glycosylation (Hickey and Buhr, 2012), deglycosylation, dimerization or degradation that could be pertinent to signaling function, the amount of every band was first corrected against additional standardized biological controls (Hickey and Buhr, 2012) and statistically analyzed.

In the present study we detected all α isoforms ($\alpha 1$, $\alpha 2$ and $\alpha 3$) in the HPM raft fraction, with $\alpha 3$ present in the largest quantity (67 % of all raft fraction α) followed by $\alpha 2$ and $\alpha 1$ (20 and 13 % respectively of all raft fraction α). Binding of ouabain to its binding site on α subunit induces

signalling (Kaplan, 2002), but the pathways activated by different α isoforms differ. In rat cardiac cells and porcine renal cells, $\alpha 1$ acts through tyrosine kinase Src that subsequently activates different signalling pathways including the Ras/Raf/ERK1/2 (Tian et al, 2006; Pierre et al, 2008) while in a mammalian cell line LM- $\alpha 3$ -1, $\alpha 3$ acts through Src-independent pathways involving PI3K and PKC (Madan et al, 2017). Downstream signalling molecules of both these pathways are known in bull sperm (Etkovitz et al, 2009; Daniel et al, 2010; Newton et al, 2010; Rotfeld et al, 2014; Rajamanickam et al, 2017). In cultured insect cells and renal cells, $\alpha 2$ does not induce signalling (Pierre et al, 2008; Xie et al, 2015), but its presence here warrants further evaluation of its role in signalling in germ cells.

Signalling is also affected by ouabain binding affinity. Significantly lower concentrations of ouabain are needed to activate signalling with $\alpha 3$ than $\alpha 1$ in insect cells (Pierre et al, 2008), which may be important given the nanomolar endogenous concentrations of ouabain in the female reproductive tract (Daniel et al, 2010). The greater amount of $\alpha 3$ in raft fractions, and its known high affinity for ouabain suggests that $\alpha 3$ may be the most likely candidate for the capacitation signal inducer in bull sperm, but none can yet be eliminated as potential stimulators of capacitation.

We also detected all the β isoforms ($\beta 1$, $\beta 2$ & $\beta 3$) in the rafts fractions of bull sperm HPM. Among different β isoforms in raft fraction, $\beta 1$ was the most prominent (69 % of all raft β), followed by $\beta 2$ and $\beta 3$ (16 and 15 % respectively of all raft β). For the Na/K-ATPase to function as a signal transducer, the α subunit should be paired with the β subunit (Liu and Askari, 2006) and among β isoforms the $\beta 1$ is widely accepted as the signalling partner with $\alpha 1/\alpha 3$ in ouabain mediated signalling in cardiac, renal and neuronal cells (Guerrero et al, 2001; Pierre et al, 2008). In addition to supporting α for proper ATPase function, β especially $\beta 1$ plays a major role in cell adhesion (Vagin et al, 2012). Since the rafts on sperm play an important role in the membrane

fusion of egg and sperm during fertilization (Reid et al, 2011; Gadella and Boerke, 2016), the β Na/K-ATPase isoforms, and specifically $\beta 1$, could play a dual role in oocyte binding and sperm capacitation. Alternatively, $\beta 1$ could be involved in adhesion and other β isoforms could be in the signalling dimers. However, engagement of $\beta 1$ in both signalling and cell adhesion would explain its more prominent presence than the other β isoforms. In summary, $\alpha 3$ and $\beta 1$ are the predominant Na/K-ATPase isoforms in the rafts of bull sperm HPM and are likely candidate isoforms for ouabain induced signalling stimulating capacitation.

Raft is a minor component of the epithelial cell plasma membrane (Simons and Toomre, 2000; García-Marcos et al, 2006), and the sperm HPM raft fractions constituted only 2.9 % of the total HPM protein. There was significantly less total Na/K-ATPase in the raft plus non-raft fractions than in the total HPM (Fig. 3.10). This paralleled the loss of all HPM protein, as only 38.8 % of the HPM protein was isolated as raft and non-raft proteins (2.9 % as raft protein and 35.9 % as non-raft protein). Most would have been lost in discarding the pellet which was always present after ultracentrifugation of HPM lysate during raft and non-raft separation, and some could have been lost during concentration of raft and non-raft proteins with Amicon® Ultra centrifugal filters.

No one isoform was restricted to the raft fractions or more concentrated in the raft fractions than another isoform ($P > 0.05$) and each of the isoforms in the raft fractions constituted <1 % of their total HPM content. Similarly rafts isolated from renal endosomes had less than 12 % of the microsomes' total ATPase content (Liu et al, 2011). But the relative prominence of certain Na/K-ATPase bands were significantly higher in the raft fraction than in the non-raft or HPM (i.e. were more enriched in raft) which could be indirect indicator of differences in function of the proteins in these bands. Since we know that the rafts are involved in signalling, enrichment of certain bands

in the raft fraction might suggest involvement of their protein(s) in signalling associated function. Our non-raft (soluble) fraction also contains all types of the Na/K-ATPase isoforms but in lesser quantity than their total HPM content, and both raft and non-raft fractions isolated from whole sperm also contained Na/K-ATPase isoforms as well as downstream signaling molecules (Rajamanickam et al, 2017). There was a loss in total HPM Na/K-ATPase after processing HPM for separating the raft and non-raft fraction. This could be part of the protein lost in the discarded pellet. The lost ATPase could be involved in signalling or other Na/K-ATPase functions like ion transport or energy metabolism.

Taken together, these findings clearly support the conclusion that no one Na/K-ATPase isoform is exclusively found in HPM raft fractions, and that the signalling pool of ATPase could be dispersed throughout the head membrane. However, rafts did contain a limited, specialized subset of bands for every isoform. These bands that preferentially located in rafts were not only from major HPM bands, and the selective raft localization of both major and minor HPM bands opens the possibility that a specialized subset of ATPase isoform proteins preferentially concentrate in rafts. Our sperm HPM was isolated from fresh, uncapacitated sperm and it is possible that capacitation would induce sequestration of specific isoforms into the rafts, although this study could not test that. Two full bovine ejaculates were required to provide sufficient HPM for analysis and subsequent raft purification, and these physical limitations prevented preparation and analysis of raft HPM from capacitated sperm.

In summary, rafts are present on the HPM of bull sperm. Although none of the different isoforms of Na/K-ATPase were unique to raft fraction, the isoforms localized in the raft fraction have unique profiles and concentrations in membrane and their presence in raft domains indicates possible involvement of these isoforms in signalling or egg-sperm interaction. Since Na/K-

ATPase-ouabain interaction is critical for inducing capacitation, knowing the exact profile of this protein may lead to better understanding of the mechanism of fertilization, and ultimately improve reproduction in domestic food animals.

4.0 INTERACTION OF OUABAIN AND PROGESTERONE ON INDUCTION OF BULL SPERM CAPACITATION

4.1 Introduction

Following deposition in the bovine female reproductive tract, spermatozoa are transported quickly through the cervix and uterus to the utero-tubal junction where they form a quiescent reserve. Functional viability of the sperm is maintained there for a species-specific period of time (Hung and Suarez, 2010). In the female reproductive tract, following the removal of seminal plasma from the surface, the sperm begins a series of reversible biochemical and physiological changes associated with capacitation, the process by which the mammalian sperm acquire their fertilizing ability (Chang, 1951; Austin, 1952). During capacitation, adsorbed decapacitating factors on the sperm plasma membrane are removed and eventually the sperm membrane progresses to the acrosome reaction (AR), finally allowing fertilization by syngamy. Various factors in the tubal fluids of female reproductive tract trigger capacitation (Rodriguez-Martinez, 2007), but the exact nature of the signal(s), and how they are received, is unknown.

Capacitation can be induced *in vitro* in ejaculated sperm in defined media containing energy substrates, a protein source, NaHCO_3 , and calcium which simulate the electrolyte composition of the oviductal fluid (Harrison, 1996; Visconti et al., 1998). Capacitation can be induced by capacitation inducing agents like heparin (Parrish, 2014) and ouabain (Thundathil et al., 2006). Ouabain is an endogenous cardiac glycoside which can also act as an endogenous steroid hormone in mammals (Schoner & Scheiner-Bobis, 2007). Ouabain also acts as a signal inducer for capacitation, interacting with Na/K-ATPase on the sperm plasma membrane, stimulating tyrosine phosphorylation (Tyr-p) of intracellular proteins to induce bull sperm capacitation (Thundathil et al., 2006; Rajamanickam et al., 2017). The Na/K-ATPase is a membrane protein which can act as

a signaling molecule in presence of its specific agonist ouabain (Wu et al., 2013). It has two main polypeptide subunits, alpha (α) and beta (β), and ouabain binds with the α subunit to exert its action (Liu and Xie, 2010; Silva and Soares-da-Silva, 2012). The α subunit has two ouabain binding sites, a low affinity binding site located between transmembrane regions (TM) 1 and TM2 and a high affinity ouabain binding site between TM4 and TM6 which differ by only a few amino acids (Sandtner et al., 2011). Ouabain is found in blood plasma and, importantly, in nanomolar concentrations in bovine semen and vaginal fluids (Daniel et al., 2010).

Progesterone (P4) is another endogenous steroid hormone that can induce capacitation and/or AR in some sperm, although the response of sperm to P4 is highly species-dependent. Progesterone does induce capacitation or AR fairly reliably in equine and human sperm (Witte and Schafer-Somi, 2007; McPartlin et al., 2008; Sagare-Patil et al., 2012), but the published responses of bovine sperm to P4 are variable. Progesterone has been reported to only induce capacitation, or only stimulate the AR in previously-capacitated sperm or induce both capacitation and AR (Therien and Manjunath, 2003; Lukoseviciute et al., 2004; 2005). The variable responses have been suggested to perhaps be due to the concentration of P4, or the physiological state of sperm, or other unknown factors.

When P4 stimulates capacitation, it is presumed to reach the sperm through follicular fluid that carries a higher concentration of P4 secreted by the cumulus cells (Baldi et al., 2009). The P4 binds to a specific receptor located on the surface of spermatozoa (membrane P4 receptor, mPR), thereby stimulating intracellular signaling (Luconi et al., 2002). Progesterone also acts as a signal transducer in amphibian oocytes, and on these oocytes P4 acts through membrane-bound Na/K-ATPase (Morrill et al., 2005). Both P4 and ouabain compete for the oocyte's low affinity ouabain binding site on the α subunit of ATPase which has structural similarity with the steroid receptor

(Seccombe et al., 1989; Morrill et al., 2005; 2008). Progesterone, which can efficiently displace ouabain from canine kidney Na/K-ATPase (Seccombe et al., 1989), has a slightly higher affinity than ouabain for the oocyte's α ATPase low affinity binding site (Morrill et al., 2008). Binding of P4 to this low affinity site stimulates a conformational change permitting caveolin to bind to the Na/K-ATPase in amphibian oocyte. The caveolin-ATPase complex then stimulates various signalling molecules including the diacyl glycerol (DAG) and MAP kinase signalling cascade (Kawamura et al., 1999, Duckworth et al., 2002, Morrill et al., 2012) which are highly reminiscent of the intracellular Tyr-p activated by ouabain in cardiac myocytes and in bull sperm for the induction of capacitation (Liu et al., 2007; Newton et al., 2010; Wu et al., 2013).

Since there is evidence that both ouabain and P4 are physiological inducers of capacitation, and both interact with Na/K-ATPase in amphibian oocyte membranes, there is a very exciting possibility that P4 and ouabain may interact at Na/K-ATPase in mammalian sperm to regulate induction of capacitation. They could potentially compete for the same binding site on the α subunit of Na/K-ATPase or act synergistically at different sites to induce bull sperm capacitation. The current work tested this hypothesis and explored the interaction (synergism/competition) of progesterone and ouabain on inducing bull sperm capacitation.

4.2 Material and Methods

4.2.1 Reagents

The following reagents were acquired from Thermo-Fisher Scientific (Unionville, ON, Canada): disodium hydrogen phosphate monohydrate ($\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$), glycine, hydrogen peroxide 30 %, methanol HPLC grade, potassium chloride (KCl), restore western blot stripping buffer, sodium bicarbonate (NaHCO_3), SDS, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, sodium vanadate, sucrose and tween

20. TEMED, 30 % acrylamide/bis solution, ammonium persulfate, precision plus protein western C standards (detect bands ranging from 10-250 kDa), precision plus strep-tactin-HRP conjugate were purchased from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada). Complete mini protease inhibitor mini cocktail tablets (EDTA free) were from Roche Diagnostics (IN, USA), percoll from GE Healthcare (Uppsala, Sweden), and Live: Dead sperm viability kit from Molecular Probes Inc. (Eugene, OR). Anti-phosphotyrosine antibody (mouse monoclonal: clone 4G10), anti- α -tubulin antibody (mouse monoclonal: clone DM1A), goat anti-mouse IgG (polyclonal), EGF-stimulated A431 cell lysate (phosphotyrosine positive control) and Immobiline-P PVDF transfer membrane (pore size 0.45 μ m) were purchased from Millipore Canada Ltd, (Etobicoke, Canada). 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, bovine serum albumin (BSA: > 96 %, essentially fatty acid free), calcium chloride (CaCl_2), dithiothreitol (DTT), EDTA, glycerol, lectin from *Pisum sativum* (FITC-PSA), luminol (97 %, HPLC grade), L- α -lysophosphatidylcholine from egg yolk (LPC), magnesium chloride ($\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$), N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)(HEPES), ouabain, paraformaldehyde (95 %), p-coumaric acid, pepstatin-A, phenyl methanesulfonyl fluoride (PMSF), poly vinyl alcohol (PVA), polyvinyl chloride (PVC), progesterone, sodium chloride (NaCl), sodium DL-lactate solution (60 % (w/w), sodium phosphate dibasic (Na_2HPO_4), sodium pyruvate, sodium vanadate, bromophenol blue, trizma base, trizma hydrochloride, carestream® kodak® autoradiography GBX fixer and replenisher, carestream® kodak® autoradiography GBX developer/replenisher, carestream® kodak® X-Omat LS film were purchased from Sigma-Aldrich, Canada Ltd (Oakville, ON, Canada).

4.2.2 Semen Collection and Evaluation

All procedures met the requirements of the University of Saskatchewan ethics and animal care requirements (Animal Use Protocol number: 20140082). Freshly ejaculated semen ($n = 9$) from Holstein bulls of varying fertility was collected at Semex (Guelph, ON, Canada) and used for the experiment. After collection, each ejaculate was examined for its initial volume, sperm motility and concentration as per routine at Semex, and the ejaculates, which had an average motility of $86 \pm 7.3\%$, was diluted with egg yolk free 'Clear extender' (proprietary composition, Semex) to a concentration of 60 Million spermatozoa/ml and transported at 18°C to the laboratory within 24 hours. The extender was designed to slow down the progressive motility of the sperm to allow it to be viable for up to 1 week at 18°C . On arrival, the extended ejaculates were warmed to room temperature (45-60 min), assessed for motility (Hamilton-Thorne motility analyzer; Version 14 HTM-IVOS) and a small aliquot was diluted to 10^7 spermatozoa per ml in PBS for assessing viability (Awda and Buhr, 2010); ejaculates with fewer than 50 % viable sperm were discarded.

4.2.3 Capacitation Assay

The various media used in the capacitation assay were prepared as previously described (Thundathil et al., 2006) with few modifications. Tyrode Albumin Lactate Pyruvate Hepes medium [Sp-TALPH; 98.3 mM NaCl, 2.99 mM KCl, 0.28 mM NaH_2PO_4 , 0.38 mM MgCl_2 , 38.4 mM Hepes, 21.0 mM lactate, 0.39 mM EDTA, 2 mM CaCl_2 , 10 mM NaHCO_3 , 1 mM pyruvate stock, PVA stock (1mg/ml); pH 6.8] was used for washing and modified Tyrode Albumin Lactate Pyruvate medium (Sp-TALP; 100.4 mM NaCl, 3.1 mM KCl, 0.28 mM NaH_2PO_4 , 1.5 mM MgCl_2 , 9.8 mM Hepes, 21.0 mM lactate, and 0.39 mM EDTA, 2 mM CaCl_2 , 24.72 mM NaHCO_3 , 1 mM pyruvate stock, 0.6 % BSA (w/v); pH 7.4) for incubating the cells. Stock solutions of both Sp-

TALP (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH₂PO₄, 1.5 mM MgCl₂, 10 mM Hepes, 21.6 mM lactate, and 0.4 mM EDTA) and Sp-TALPH (Sp-TALP with 0.4 mM MgCl₂, 40 mM Hepes) were prepared and stored at -20°C and working solutions were prepared on the day of experiment. Stock solution of P4 (20 mM) was prepared in absolute ethanol and stored at -20°C while working solutions (50 µM) were prepared in Sp-TALP on the day of experiment. The ouabain was freshly prepared in Sp-TALP (20mM stock and 50µM working solution). Since both ouabain and P4 were light sensitive, these chemicals were prepared and stored in dark.

For the capacitation assay, the extended ejaculate was warmed to room temperature and the extender removed by centrifugation (800 x g, 10 min, 25°C) and the pellet resuspended with Sp-TALPH (1:5, vol:vol). The diluted sperm were placed on a two-step (45 % - 90 %) percoll gradient (1:1:1, vol:vol:vol), centrifuged (700 x g, 25 min, 25°C) and the pellet washed once with Sp-TALPH and then with Sp-TALP (380 x g, 30 min, 25°C). The final sperm concentration was adjusted to 100 x 10⁶ sperm/ml with Sp-TALP. To induce capacitation, identical aliquots of prepared sperm with various treatments (5 x 10⁶ sperm in a final volume of 100 µl) were incubated in a small round bottomed glass tube in the dark for 5 hours (39°C, 5 % CO₂ and high humidity) with gentle agitation every hour. All treatments and materials were in Sp-TALP.

4.2.4 Onset of AR and Tyr-p of Sperm Proteins

To compare the onset of AR in whole sperm and Tyr-p in proteins from whole sperm, two identical aliquots of sperm from each ejaculate (n = 5 bulls) were incubated with capacitation medium alone (control), or with 50 µM ouabain, 50 µM P4 or 25 µM ouabain plus 25 µM P4 (Ou+P4) under ideal capacitating conditions as described above. Samples were taken at 0 and 5

hr. At each time point, one aliquot was used to microscopically evaluate capacitation status of sperm and the second to evaluate Tyr-p of proteins.

To measure capacitation microscopically (by assessing acrosome reacted sperm as only capacitated sperm can undergo AR), 100 μ l of sperm suspension in one of the identical aliquots was divided into two equal parts (50 μ l each) in two separate round bottomed glass tubes, adding 50 μ l LPC (100 μ g/ml in PBS) to one part and 50 μ l Sp-TALPH to the other part and incubated again (30 min, 39°C, high humidity) to induce AR. After incubation, 25 μ l of sperm suspension was gently smeared on a glass microscope slide, air dried, fixed in absolute ethanol (-20°C for 5 minutes) and stained with FITC-conjugated *Pisum sativum* agglutinin (PSA) for 5 minutes. After washing, the acrosomal status of sperm was determined (2 x 100 spermatozoa/slide; assessed by one trained observer who was blind to the treatments) and spermatozoa were classified as having an intact acrosome, or damaged acrosome, or lost acrosome (Fig. 4.1; Herold et al., 2004; Thundathil et al., 2006).

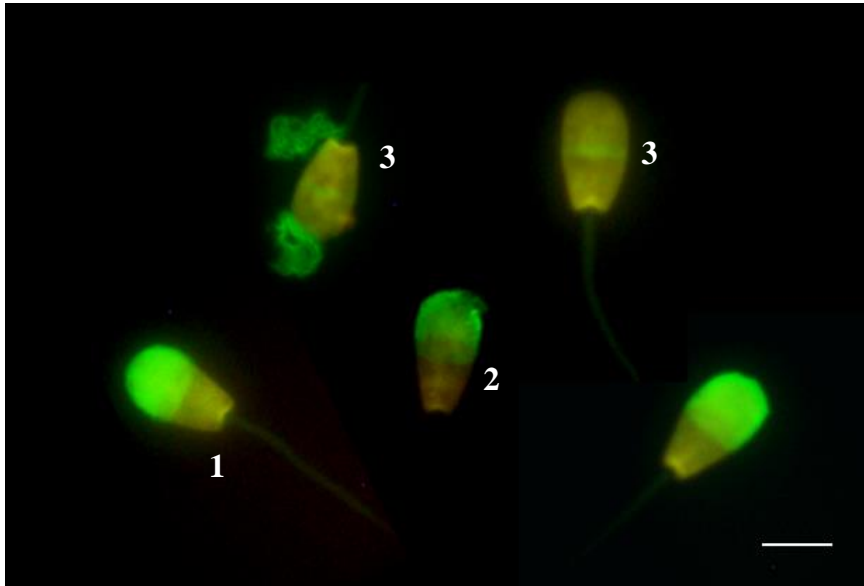


Figure 4.1: Acrosomal status of bovine sperm. Typical fluorescent images of bull spermatozoa after staining with FTTC-PSA (40x). Spermatozoa were classified as having: 1- intact acrosome, 2- damaged acrosome and 3- reacted acrosome. Scale bar represents 5 micrometers.

To measure Tyr-p, proteins were extracted from the second aliquot of incubated sperm by washing in 1 ml of PBS containing 0.2mM Sodium vanadate ($10000 \times g$, 5 min, $25^{\circ}C$) and the final pellet resuspended in 50 μl of 0.2mM Sodium vanadate containing protease inhibitor (mini cOmplete™ Protease Inhibitor Cocktail). This was snap frozen in liquid nitrogen and stored at $-80^{\circ}C$ overnight for western immunoblotting (WB) on the next day. The WB was carried out as before (Thundathil et al., 2006; Hickey and Buhr, 2012): the sample was thawed (on ice for 30 minutes, then room temperature), boiled in sample buffer ($100^{\circ}C$, 5 minutes; 7.5% dithiothreitol (w/v), 10.8% SDS (w/v), 43% 1M pH 6.8 Tris (v/v), 53.8% of 50% glycerol (v/v) and 3.2% of 2.5% bromophenol (v/v)), cooled to room temperature, centrifuged ($10000 \times g$, 5 min) and 25 μl of the resultant supernatant was layered on an SDS-PAGE gel (4 % stacking and 10 % running

polyacrylamide gel); gels also included manufacturer's positive controls. Gels were subjected to electrophoresis at 75V (15 min, room temperature; Stacking gel) followed by 100V (90 minutes, room temperature; running gel). Separated protein bands were electrotransferred (100V; 1 h; 4°C) on to PVDF membranes (pore size 0.45 µm) in tris/glycine buffer (250 mM Tris, 1.92 M glycine, pH 8.3) and 20 % methanol. After blocking nonspecific binding sites with 5 % (w/v) skim milk blocking buffer for 1 hour, membranes were incubated with anti-phosphotyrosine antibody (1:12500 in TTBS (20mM tris-buffered saline containing Tween 20) for two hours followed by secondary antibody (1:2500 goat anti-mouse IgG in TTBS) for one hour. The sensitivity of the primary antibody was verified against commercial positive controls in every replicate and also by incubating membranes with secondary antibody alone. The bands from the extracted proteins were detected with chemiluminescence (incubating the membranes in 50 µl 68 mM p-coumaric acid, 5 ml 1.25 mM luminol and 15 µl 3 % peroxide for 2 minutes) and images were developed using Kodak scientific imaging films (Caraestream Health, Inc., Rochester, NY, USA). The bands from the extracted proteins from each treatment at each time point were analyzed separately for Tyr-p. Visible bands were assigned a molecular weight (kDa) by comparison against molecular weight standards on each gel using Image Quant TL software (Ver 8.1; GE Healthcare Bio- Science Inc., Baie d'Urfe, QC, Canada). Each band was quantified for its volume (sum of the pixel intensity for all pixels in a given selection) and area (number of pixels in a given selection). Amount of each MW band was corrected for gel to gel variation based on intensity of the positive control bands.

4.2.5 Effect of Ouabain, P4 and their Combination on Tyrosine Phosphorylation of Sperm Proteins Associated with Capacitation

To assess the impact of P4, ouabain and their interaction on induction of capacitation, three identical aliquots of sperm (n = 9) were incubated in capacitation medium alone (control) or that

medium containing ouabain (Ou; at 12.5, 25, 50 and 100 μ M final concentration) or P4 (12.5, 25, 50 and 100 μ M final concentration) or combined ouabain plus P4 (Ou+P4; each at 12.5, 25 and 50 μ M final for each steroid) and the amount of phosphorylated tyrosine assessed by WB at 0, 3 and 5 hours of incubation. The P4 was dissolved in ≤ 0.1 % absolute ethanol which is safe for bull sperm (Therian and Manjunath, 2003). The effect of higher concentrations of P4 on cell viability was determined by comparing P4 to the control treatments at each time point using Live and Dead sperm viability kit (Molecular Probes Inc., Eugene, OR), counting 2×100 spermatozoa as live (green) or dead (red) cells using a fluorescent microscope (Laborlux S; Leitz, Germany) fitted with a blue filter under 40x objective. After detecting the Tyr-p protein bands as previously described, the membranes were stripped (37°C, 30 minutes, restore western blot stripping buffer; Thermo-Fisher Scientific, Alberta, Canada) and reprobed with anti- α -Tubulin Antibody (1:5000 in TTBS) to confirm protein load (Awda and Buhr, 2010).

4.2.6 Data Analysis

All data were checked for normality, corrected as appropriate by log transformation and statistical analyses performed on the normalized data. All the data were analyzed using Mixed Model Analysis of Statistical Analysis Software (SAS; version 9.4; SAS Institute, Inc., Cary, NC). The percentage of spermatozoa undergoing AR (% of damaged sperm + % acrosome reacted sperm) induced by LPC (only capacitated sperm undergo AR with LPC) was measured and corrected for spontaneous AR by subtracting the percentage of acrosome reacted spermatozoa present in the samples incubated in Sp-TALPH (no LPC) and this corrected data was used for the analysis. Data for onset of AR, as well as the logarithmic transformed normalized band volume for the same experiment were analyzed with a randomized complete block design (RCBD) with

treatment as fixed effect and day of experiment as block. Mean separation was done using Tukey's test. For analyzing the impact and interaction of ouabain and P4, the actual band volume data after normalization by logarithmic transformation were analyzed as RCBD with repeated measures with the effect of treatment \times time included in the model (assuming replicate tubes in any one ejaculate were identical). The covariance model with lowest Akaike's information criterion (AIC) or Bayesian information criterion (BIC) values was selected for the repeated measure analysis (Littell et al., 1996). For this same experiment, the actual increase in volume and the percentage increase for the total volume of selected bands over 3 and 5 hours were analyzed as RCBD with 12 (treatment) \times 2 (increase in volume over 3 and 5 hours) factorial arrangement of treatments.

4.3 Results

Even after 24 hours of shipping, the extended ejaculates had an average motility of 82 ± 7.55 % on arrival. After washing to remove extender and kept at 37°C for 3 hour, the individual ejaculates used in this study had 78 ± 11.6 % viable and 72 ± 9.0 % motile sperm (mean \pm SE).

4.3.1 Onset of AR and Tyr-p of Sperm Proteins

Only sperm incubated in $50\text{ }\mu\text{M}$ ouabain for 5 hours showed a significant increase in Tyr-p proteins, compared to either the amount at 0 hours or any other treatment (Fig. 4.2 and Fig. 4.3A; Appendix 2.1). Microscopic assessment of sperm induced to acrosome react with LPC showed similar responses, with only 5 hours of incubation in $50\text{ }\mu\text{M}$ ouabain allowing a significant proportion of sperm to capacitate in the presence of LPC (Fig. 4.3B; Appendix 2.2). Sperm incubated for 5 hours in the combined $25\text{ }\mu\text{M}$ ouabain plus $25\text{ }\mu\text{M}$ P4 did show a tendency to increased capacitation compared to 0 hours ($P = 0.08$).

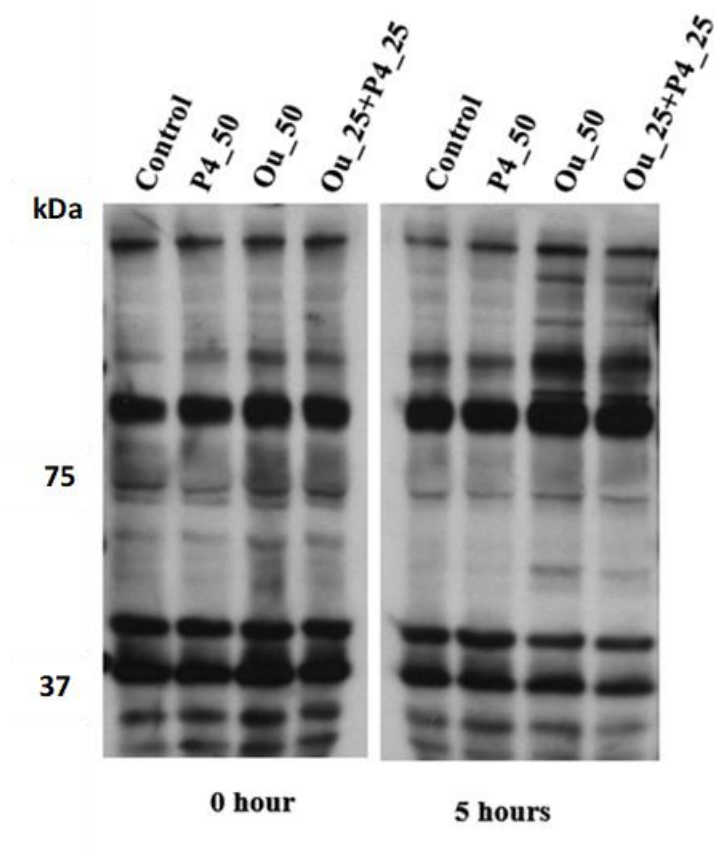


Figure 4.2: Western immunoblot of tyrosine phosphorylated proteins from whole bull sperm. Bull sperm were incubated for 0 and 5 hours in Sp-TALP (5×10^6 sperm per 100 μ l) with ouabain (Ou) or progesterone (P4) at 0 (control) or 50 μ M or 25 μ M each (P4+Ou). Proteins were extracted at each time point, gel was run and detected the tyrosine phosphorylated proteins band with anti-phosphotyrosine antibody

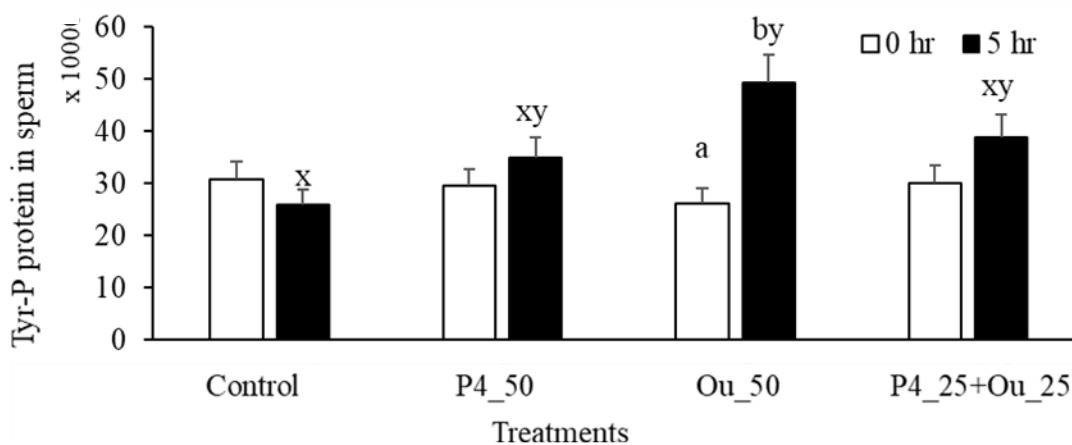
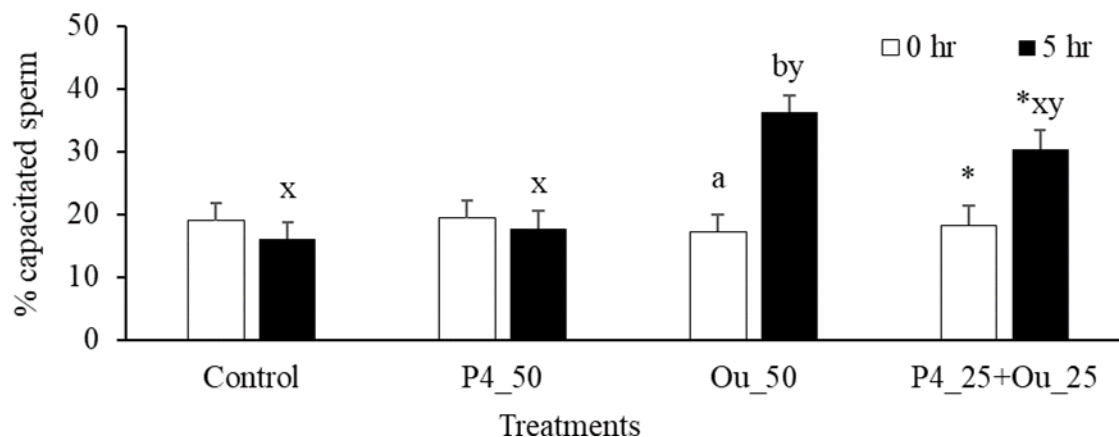
A**B**

Figure 4.3: Capacitation of bovine sperm exposed to ouabain and progesterone. Sperm ($n = 5$ ejaculates) were incubated for 0 and 5 hours in Sp-TALP (5×10^6 sperm per $100 \mu\text{l}$) with ouabain (Ou) or progesterone (P4) at 0 (control) or $50 \mu\text{M}$ or $25 \mu\text{M}$ each (P4+Ou). (A) Total amount of tyrosine phosphorylated (Tyr-p) proteins per 5×10^6 sperm, calculated as the total pixel intensity in Image-Quant analysis of all Tyr-p protein bands. (B) Microscopic detection of sperm acrosomal status. After the indicated incubations, sperm were exposed to 0 or $5 \mu\text{g}$ LPC for 30 minutes and observed microscopically for acrosomal status. % capacitation is the difference in the number of acrosome-reacted sperm in the presence and absence of LPC. All values are means \pm SEM and all analyses were performed on normalized data. Data was analyzed as randomized complete block design (RCBD) and the mean comparison was done by Tukey's test. a-b Times 0 and 5 hours differ within a treatment, $P < 0.05$. * Times 0 and 5 hours tend to differ, $P = 0.08$. x-y at 5 hours, treatments without a common letter differ, $P < 0.05$. Treatments at 0 hours did not differ; superscripts are omitted for ease of viewing.

4.3.2 Tyr-p of Sperm Proteins

There were 15 immunoreactive bands of Tyr-p proteins (214 ± 5.8 , 182 ± 11.3 , 104 ± 10.2 , 81 ± 4.7 , 58 ± 3.0 , 51 ± 0.8 , 47 ± 1.5 , 40 ± 1.0 , 36 ± 0.8 , 31 ± 0.7 , 28 ± 0.7 , 26 ± 0.7 , 24 ± 0.9 , 22 ± 0.6 and 17 ± 1.0 kDa; Fig. 4.4). The equal amount of protein loaded into the wells of SDS gels for Tyr-p immunoblots were confirmed by alpha-tubulin density. Analysis of the amount (volume) of each band at each treatment and incubation time found 7 bands to significantly increase in intensity during incubation (104 ± 10.16 , 81 ± 4.71 , 58 ± 2.95 , 47 ± 1.47 , 31 ± 0.69 , 28 ± 0.69 , and 17 ± 1.01 kDa; $P < 0.05$). The 17kDa band was the only band to increase during incubation with the control media. The other six bands increased somewhat variably when sperm were incubated with different treatments and incubation times but did not increase in control media and all increased with some doses of ouabain. Of the seven protein bands which are significantly phosphorylated during ouabain-induced capacitation, P4 phosphorylates only three (104, 47 and 17 kDa), and did not phosphorylate any that are not phosphorylated in response to ouabain. These seven bands were therefore considered the capacitation-sensitive protein bands.

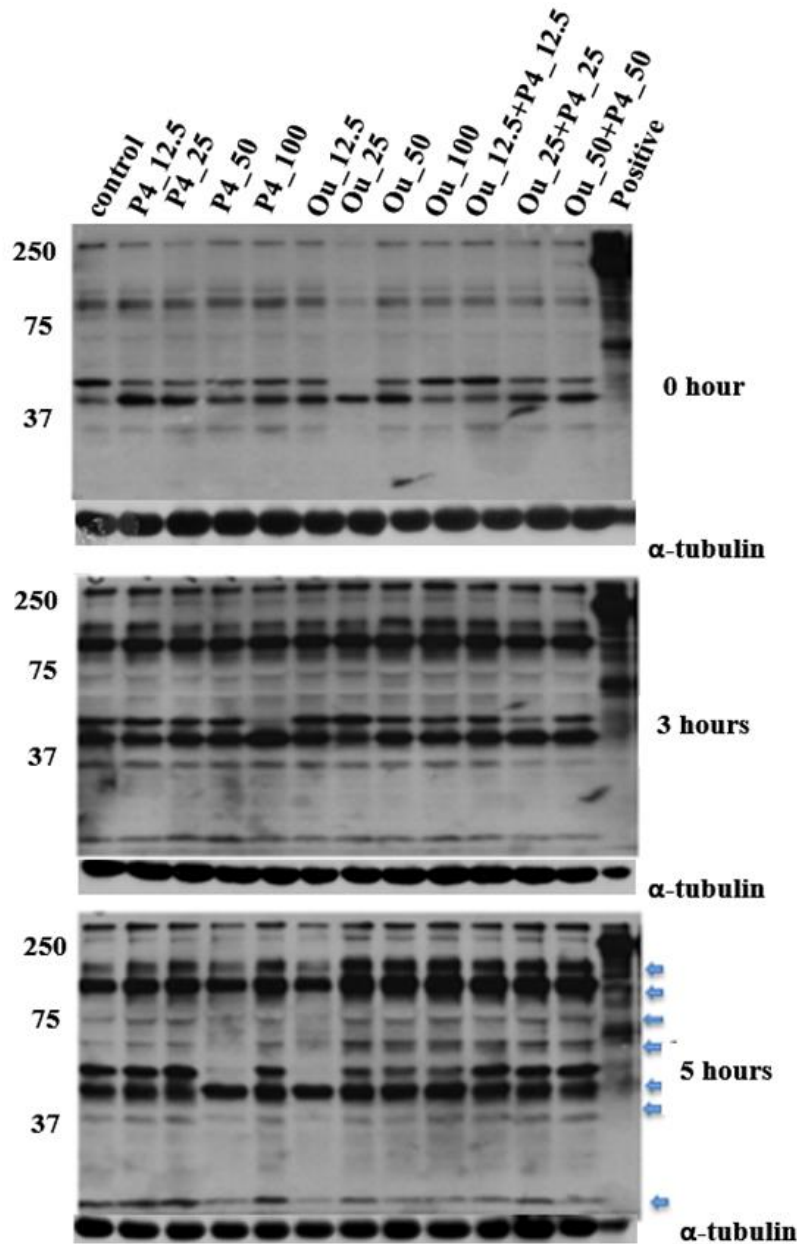


Figure 4.4: Western immunoblot of tyrosine phosphorylated proteins from whole bull sperm.

Bull sperm were incubated for 0, 3 and 5 hours in Sp-TALP (5×10^6 sperm per $100 \mu\text{l}$) with ouabain (Ou) or progesterone (P4) at 0 (control), 12.5, 25, 50 and $100 \mu\text{M}$ or 12.5, 25 and $50 \mu\text{M}$ each (P4+Ou). Proteins were extracted at each time point, gel was run and detected the tyrosine phosphorylated protein band with anti-phosphotyrosine antibody. EGF-stimulated A431 cell lysate (lane 13, $15 \mu\text{g}$ protein loaded) was the manufacturer positive control. Arrowheads at the right of the 5 hours immunoblot indicate the protein bands (104 ± 10.16 , 81 ± 4.71 , 58 ± 2.95 , 47 ± 1.47 , 31 ± 0.69 , 28 ± 0.69 , and 17 ± 1.01 kDa) which significantly phosphorylated over time with different treatments. After detecting the tyrosine phosphorylated protein bands, the blot from each time point was stripped and reprobed with anti- α -tubulin antibody to confirm protein load.

4.3.3 Effect of Ouabain, Progesterone and their Combination on Capacitation

Viability of sperm incubated for 5 hr with varying doses of P4 was similar to control sperm incubated in media alone (control Vs P4 at 12.5, 25, 50, 100 μ M; 48 vs 46, 44, 45, 42 %, all \pm SEM 2.5 %). Viability of ouabain-treated sperm was not tested in this experiment as it is already documented that the viability of bull sperm is not affected by 10-100 μ M ouabain under the same experimental conditions (Thundathil et al., 2006; Newton et al., 2010).

Overall, both ouabain (all doses pooled) and P4 significantly increased total Tyr-p of the sensitive proteins after 3 and 5 hours of incubation, with more phosphorylation at 5 hours ($P < 0.05$, Fig. 4.5A; Appendix 2.3). Ouabain and P4 combined doses also caused similar overall effects to those of the steroids separately (overall total Tyr-p, 5 hr $>$ 3 hr $>$ 0 hr; $P < 0.05$). But at 5 hours, only ouabain induced significantly more Tyr-p of sperm protein than the control medium while the increase by P4 or mixed steroids was not different from the control sperm ($P > 0.05$). For all the treatments except control more proteins were phosphorylated during later stages of incubation ($P < 0.05$, Fig. 4.5B; Appendix 2.4). Among different treatments, the actual increase in Tyr-p from zero hours did not differ among the three pooled treatment groups (ouabain, P4 and Ou+P4) at 3 hours. However, incubation for 5 hours with ouabain alone and the mixed steroids induced a significantly higher actual increase than did either control or P4 alone, but the actual increase by mixed steroid was significantly less than the increase by ouabain alone ($P < 0.05$, Fig. 4.5 B; Appendix 2.4).

Even though both P4 and ouabain overall stimulated an increase in total Tyr-p (band intensity) over all times, the individual doses did cause differential responses. Sperm incubated with 100 μ M P4, 12.5, 25, 50 and 100 μ M ouabain and 12.5, 25 and 50 μ M Ouab+P4 showed an intermediary, non-significant increase in Tyr-p from 0 to 3 hours of incubation, and that increase

became significant after 5 hours of incubation ($P < 0.05$, Fig. 4.6 A; Appendix 2.5). Only incubation for 5 hours in 12.5, 25, 50 and 100 μM ouabain and 12.5 μM Ouab+P4 induced significantly more Tyr-p than in the control medium ($P < 0.05$). Among different treatments, the actual increase in Tyr-p proteins induced by 50 μM ouabain was significantly higher during later stages of incubation while other treatments maintained the rate of increase during both early and later stages. (Fig. 4.6 B; Appendix 2.6). Note that 5 hours of exposure to 25 and 50 μM ouabain stimulated a significant increase in Tyr-p compared to control, but these same doses of ouabain in the presence of P4 did not induce a significant increase compared to control (Fig. 4.6 B).

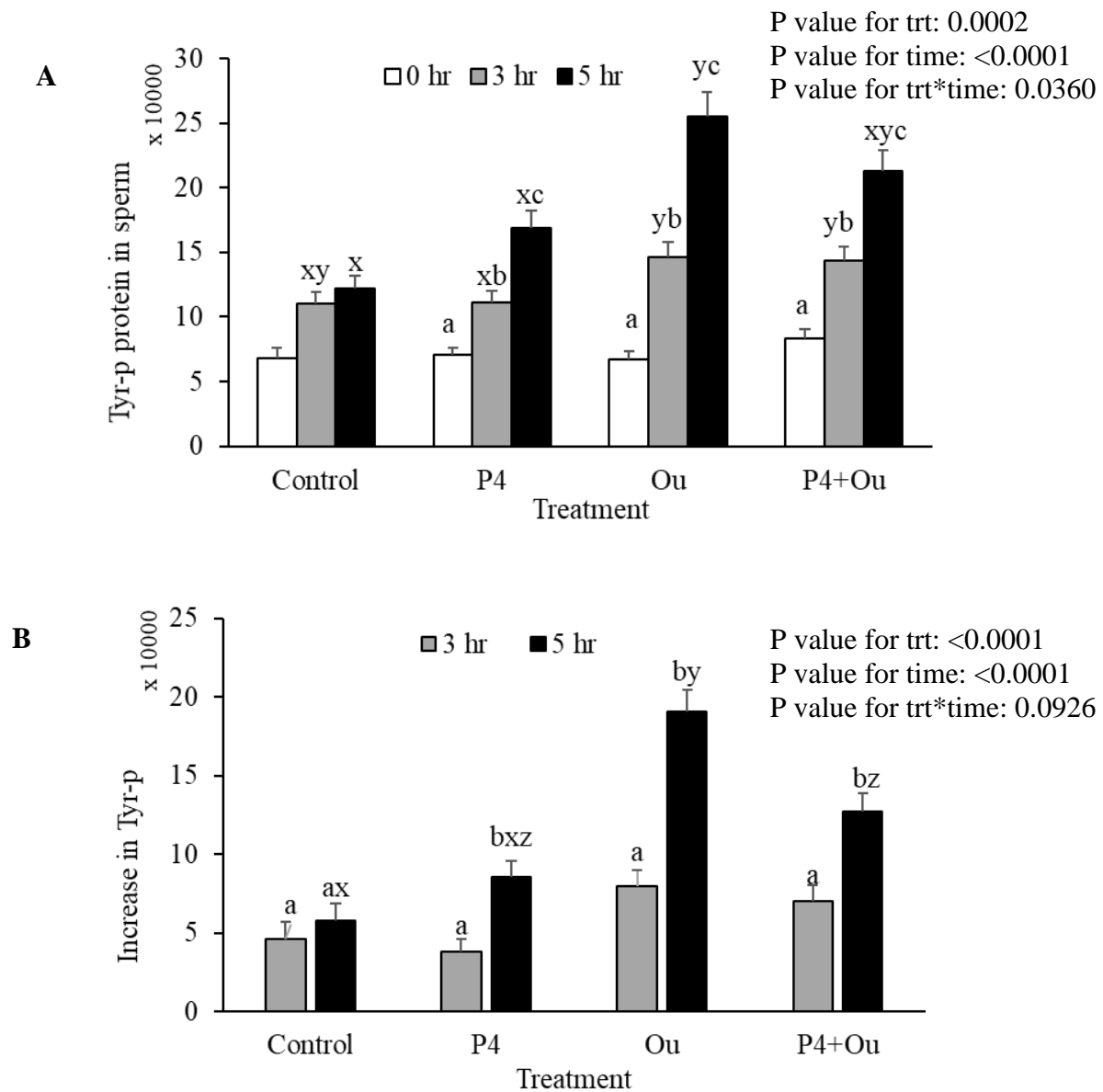


Figure 4.5: Protein tyrosine phosphorylation in bull sperm induced by overall doses of ouabain, progesterone and ouabain+ progesterone under capacitating conditions. Sperm ($n = 9$ ejaculates) were incubated for 0, 3 and 5 hours in Sp-TALP (5×10^6 sperm per $100 \mu\text{l}$) with ouabain (Ou) or progesterone (P4) at 0 (control), 25, 50 and $100 \mu\text{M}$ or 12.5, 25 and $50 \mu\text{M}$ each (P4+Ou); pooled data from three doses for Ou and P4 (25, 50, $100 \mu\text{M}$) and Ou+P4 (each at 12.5, 25 & $50 \mu\text{M}$) were used. (A) Total amount of tyrosine phosphorylated (Tyr-p) proteins per 5×10^6 sperm calculated as the total pixel intensity of Tyr-p bands which increased in intensity over time in Image-Quant analysis. Data was analyzed as randomized complete block design (RCBD) with repeated measures and the mean comparison was done by Tukey's test (B) Calculated increase in amount of Tyr-p proteins over 3 and 5 hours. Data was analyzed as RCBD and the mean comparison was done by Tukey's test. a-c Times 0, 3 and 5 hours differ within treatment, $P < 0.05$; x-y at 3 and 5 hours, treatments without a common letter differ, $P < 0.05$. Treatments at 0 hours did not differ; superscripts are omitted for ease of viewing.

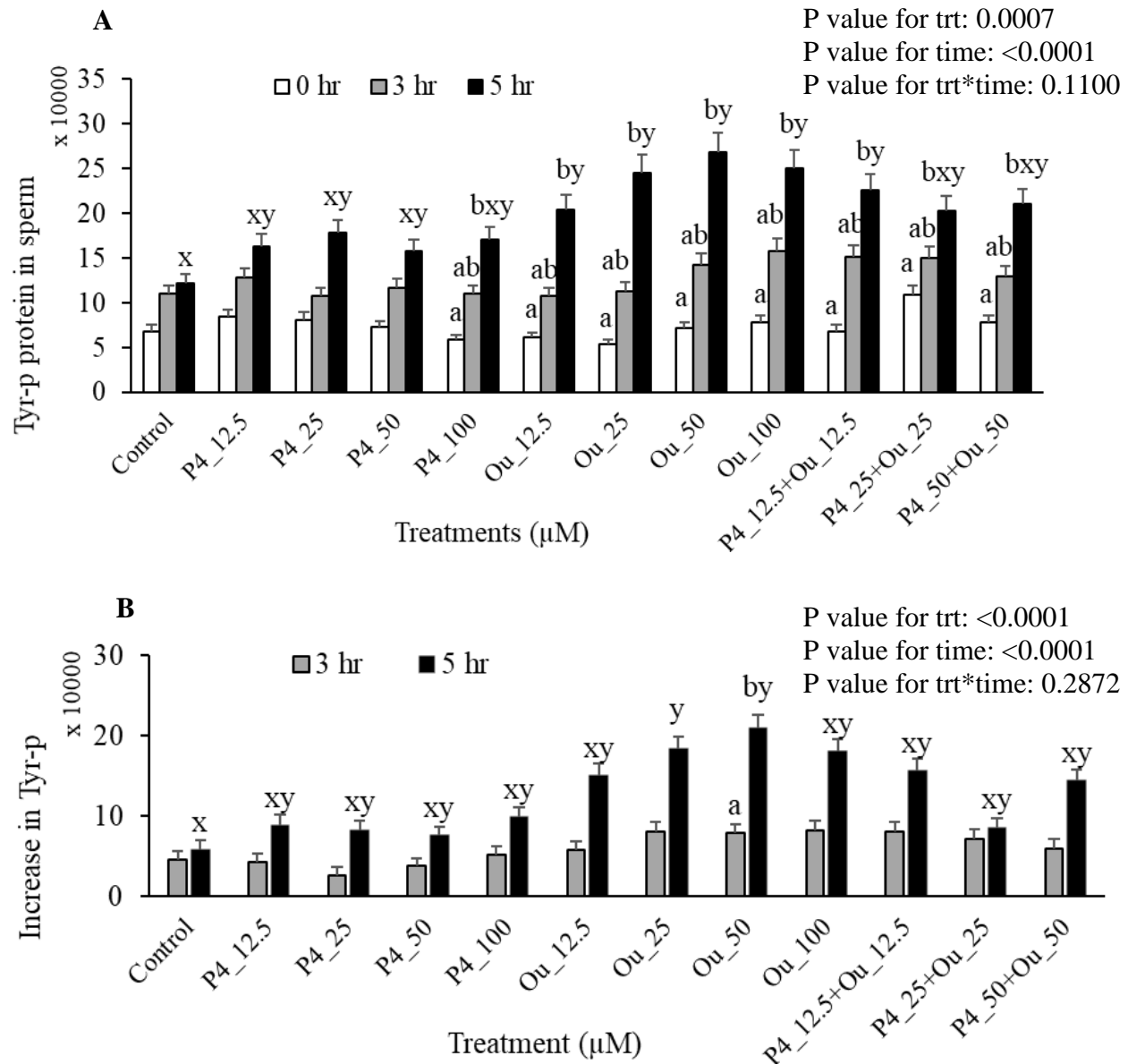


Figure 4.6: Protein tyrosine phosphorylation of bull sperm exposed to various doses of ouabain, progesterone and ouabain+ progesterone and incubated under capacitating conditions. Sperm ($n = 9$ ejaculates) were incubated for 0, 3 and 5 hours in Sp-TALP (5×10^6 sperm per $100 \mu\text{l}$) with ouabain (Ou) or progesterone (P4) at 0 (control), 12.5, 25, 50 and $100 \mu\text{M}$ or 12.5, 25 and $50 \mu\text{M}$ each (P4+Ou) (A) Total amount of tyrosine phosphorylated (Tyr-p) proteins per 5×10^6 sperm calculated as the total pixel intensity of Tyr-p bands which increased in intensity over time in Image-Quant analysis. Data was analyzed as randomized complete block design (RCBD) with repeated measures and the mean comparison was done by Tukey's test (B) Calculated increase in amount of Tyr-p proteins over 3 and 5 hours. Data was analyzed as RCBD and the mean comparison was done by Tukey's test. a-b Times without a common letter differ within treatment, $P < 0.05$. x-y at 5 hours, treatments without a common letter differ, $P < 0.05$. Treatments at 0 hours did not differ; superscripts are omitted for ease of viewing.

4.4 Discussion

For the first time, ouabain and P4 have been shown to interact in bull sperm capacitation. While both steroids stimulated Tyr-p of certain sperm proteins, ouabain phosphorylated more proteins, caused significantly more total Tyr-p and more efficiently stimulated onset of the AR than P4 did, and P4 partially interfered with ouabain-induced responses possibly by replacing ouabain from the low affinity ouabain binding site on Na/K ATPase. These results cast light on the mechanisms by which P4 and ouabain interact with their binding site to induce signaling pathways in bovine sperm capacitation.

Under *in vivo* conditions, bull sperm are exposed to nM concentrations of ouabain and P4 in the female reproductive tract (Daniel et al., 2010; Lamy et al., 2016). But this experiment used μ M concentrations of ouabain and P4 which is documented to be able *in vitro* to activate several downstream signaling pathways leading to the Tyr-p of various sperm proteins without inhibiting the normal physiological functions of sperm under *in vitro* conditions (Lukoseviciute et al., 2004; Thundathil et al., 2006; Newton et al., 2010). Even though increase in intracellular concentration of Na⁺ ions are associated with capacitation of mammalian sperm (Yanagimachi, 1994), ouabain-induced capacitation is unlikely to change the ionic balance of sperm as these concentrations of ouabain neither stimulate nor inhibit Na/K-ATPase enzymatic activity (Thundathil et al., 2006).

One of the major molecular responses associated with capacitation is Tyr-p of sperm proteins (Signorelli et al., 2012; Campbell et al., 2013). Since mature sperm are devoid of any major transcriptional and translational activity, post-translational modifications such as phosphorylation are the major means of obtaining proteins with modified function to achieve fertilization (Brohi and Huo, 2017). The initial experiment of this study first confirmed that the semi-quantitative measurement of Tyr-p of sperm proteins accurately reflect and directly parallels

capacitation and onset of AR as shown before (Thundathil et al., 2006), and it also clearly demonstrated for the first time that P4 interferes with the known efficacy of 50 μ M ouabain in inducing capacitation of bull sperm.

Seven protein bands were significantly phosphorylated following ouabain-induced bull sperm capacitation indicating their potential involvement in the process of capacitation. Similar molecular weight proteins have previously been shown to be phosphorylated in bull sperm capacitation (Thundathil et al., 2006). These capacitation-associated protein bands are considered to be the critical proteins in the signalling pathway for capacitation, and their molecular weights are consistent with some major proteins involved in bull sperm capacitation. These include serine/threonine-protein phosphatase PP1- α catalytic subunit (38 kDa), membrane metallo-endopeptidase-like 1 (~ 90 kDa), A-kinase anchor protein 4 (AKAP4, 95 kDa), major fibrous sheath protein (82 kDa), outer dense fiber protein 2 (55 kDa), outer dense fiber of sperm tails 2 (84 kDa), succinate dehydrogenase (ubiquinone) flavoprotein subunit (70-73 kDa) and dihydrolipoyl dehydrogenase (DLD ~ 70-73 kDa). They also have been implicated in the processes of hyperactivation, AR and ultimately bovine fertilization (Jagan Mohanarao and Atreja, 2011; Chauhan et al., 2018).

Exposure to ouabain stimulated significantly increased phosphorylation of all seven protein bands while P4 phosphorylated only three of the same protein bands. Certainly P4's lesser phosphorylation stimulatory effect cannot be attributed to any harmful effect of P4 on sperm function as the viability of sperm was not affected by 12.5 -100 μ M P4. Therefore, it seems likely that ouabain and P4 activate the same pathways, but that ouabain either stimulates more downstream signaling steps than P4 does, or that ouabain also stimulates one or more additional pathway(s). The latter seems probable, since binding of ouabain to the α subunit of Na/K-ATPase

stimulates signalling cascades involving cAMP/PKA, Src/RTK/PLC/IP3/DAG/PKC pathway and MAPK pathway leading to Tyr-p of proteins and capacitation of bull sperm (Newton et al., 2010; Rajamanickam et al., 2017), while binding of P4 to the low affinity ouabain binding site on the α subunit of Na/K-ATPase in amphibian oocytes induces a signalling cascade involving DAG (Morrill et al., 2010) but not cAMP/PKA. Similarly, the various signalling pathways reported to be induced by P4 during capacitation in various species involved Ca^{2+} induced PLC-DAG/IP3-PKC and MAPK pathways, but not cAMP/PKA (Luconi et al., 1998; Thundathil et al., 2002; Ickowicz et al., 2012; Jin and Yang, 2016).

As noted, ouabain, but not P4, induced an overall increase in Tyr-p of bull sperm proteins after 5 hours of incubation, (ouabain > P4, 298 % > 137 %; $P < 0.05$; Fig. 4.5 & Appendix 2.4). Ouabain also significantly increased capacitation, measured as LPC-induced acrosome reaction, while again P4 did not (Fig. 4.3 B). Moreover, among individual treatments only 25 and 50 μM ouabain significantly phosphorylated more proteins after 5 hours than control, and only 50 μM ouabain significantly increased Tyr-p from 3 to 5 hr (Fig. 4.6 B). These results clearly indicate that ouabain induces capacitation more effectively than P4.

Furthermore, the presence of P4 reduced ouabain-induced Tyr-p, whether in comparing the same total dose of steroid (ouabain 25 μM to P4+ouabain 12.5 μM each; ouabain 50 μM to P4+ouabain 25 μM each), or in comparing the same total amount of ouabain (ouabain 25 μM to P4+ouabain 25 μM each; ouabain 50 μM to P4+ouabain 50 μM each). This P4 partial inhibition on ouabain action supports the hypothesis that ouabain and P4 compete for the same binding site in bull sperm. In amphibian oocytes, ouabain and P4 compete for the low affinity ouabain binding site on the $\alpha 1$ subunit of Na/K-ATPase which has a structural similarity with the steroid receptor (Morrill et al., 2005; 2008). Since ouabain and P4 are both steroids, they could compete for similar

receptors and bind to the low affinity ouabain binding site through their steroid core. Indeed, P4 apparently has slightly higher affinity than ouabain for this low affinity ouabain binding site and hence, when ouabain and P4 are mixed together, the P4 would preferentially occupy the low affinity site, at least partially replacing ouabain-induced signaling by P4 induced signalling (Seccombe et al., 1989; Morrill et al., 2008). Since P4 induced signaling is less effective at phosphorylating tyrosine, the overall Tyr-p would be reduced. Even though the two low and high affinity binding ouabain sites on the α subunit of Na/K-ATPase have not been studied well in mammalian sperm, the current results clearly support their presence on bull sperm and P4 appears to partially or completely replace ouabain from its low affinity binding site resulting in a reduced overall Tyr-p.

Various molecules have been identified as mPRs on mammalian sperm (Luconi et al., 1998; Jang and Yi, 2005; Thomas et al., 2005), but the exact nature of these molecules have not been well documented on bull sperm. It is also possible that P4 can act through these molecules to exert its action on sperm capacitation. Since both ouabain and P4 are steroids, it is possible that ouabain can also act through these mPRs to exert its action. Since it is already established that Na/K-ATPase is the specific receptor for ouabain, partial inhibition of ouabain action by P4 supports the possibility that P4 and ouabain can act through the same binding site on Na/K-ATPase. Interestingly, the confirmed presence of low and high affinity ouabain binding sites on the amphibian oocyte $\alpha 1$ isoform of Na/K-ATPase, may imply there are more than one ouabain binding sites on the other α isoforms known in bull sperm (Hickey and Buhr, 2012). The precise role of α isoform involved in capacitation induction in bull sperm is unknown, and certainly in other cell types the $\alpha 2$ and $\alpha 3$ isoforms can induce specific signalling after binding to ouabain (Zhang et al., 2008; Reinhard et al., 2013; Peng et al., 2016).

The previous studies showing inconsistent stimulatory effects of P4 on inducing ovine sperm capacitation (Therien and Manjunath, 2003; Lukoseviciute et al., 2004; 2005) may be due to P4 not being the primary stimulus. Since ouabain is the most efficient capacitation phosphorylating agent and P4 appears to phosphorylate proteins only through its limited activation of a portion of ouabain's stimulatory pathways, it seems reasonable that ouabain is the primary stimulus for inducing bovine capacitation in this study, and P4 would be expected to only ineffectively partially duplicate the major effector. But more studies are needed to understand the exact mechanism involved in the interaction of ouabain and P4 in bull sperm and the functional importance of this interaction on regulating bull sperm functions in the female reproductive tract.

In conclusion, while both ouabain and P4 induce Tyr-p of certain bull sperm proteins and apparently interact with the same binding site on Na/K-ATPase in this study, ouabain appears to be the more effective, and therefore possibly the primary, physiological inducer of bovine capacitation.

5.0 OUABAIN AND PROGESTERONE INDUCED CAPACITATION IN SPERM FROM BULLS OF VARYING FERTILITY

5.1 Introduction

Bull fertility plays a key role in animal breeding as the male contributes half of the genetic composition to the next generation. Fertility of spermatozoa is not only related to motility, morphology or other structural characteristics like integrity of plasma membrane or DNA but also closely related to various molecular events happening in spermatozoa which aids in interacting with the oocyte plasma membrane (Berger et al., 1996). Spermatozoa from males of good field fertility have generally high overall functional competence (Petrunka et al., 2007), but there has been little success in identifying laboratory tests of spermatozoa that correlate with a male's *in vivo* fertilizing success. Capacitation is one of the major molecular events every mammalian sperm needs to undergo to fertilize the oocyte, and timely capacitation of spermatozoa is necessary for successful fertilization (de Lamirande et al., 1997b). Capacitation can be induced *in vitro* (Parrish et al., 1988) using chemicals that are often species-specific. Heparin is widely accepted as a capacitation inducing agent of bull sperm, but its binding affinity for sperm is not consistently correlated with fertility (Ax and Lenz, 1987; Merkies et al., 2000; Karunakaran and Devanathan, 2017). Progesterone (P4) is a steroid hormone that induces capacitation in equine and human sperm but is inconsistent in inducing capacitation in bull sperm (Therien and Manjunath, 2003; Lukoseviciute et al, 2004; 2005). Ouabain is an endogenous cardiotonic steroid that we have shown induces capacitation of bull sperm (Thundathil et al., 2006) by acting as a steroid hormone binding to Na/K-ATPase in the plasma membrane of the sperm head (HPM) to induce signalling. Ouabain binds to highly specific binding sites on the α subunit of Na/K-ATPase to stimulate capacitation through Tyr-p of sperm proteins. Progesterone can also bind to the α subunit of Na/K-

ATPase to induce signalling in frog oocytes (Morrill et al., 2005; 2008). This opens the possibility that both ouabain and P4 interact to induce Tyr-p in bull sperm (Chapter 4). The present study compares the interaction of ouabain and P4 on inducing bovine sperm capacitation in a manner correlated to field fertility.

5.2 Materials and Methods

All chemicals used, except those specified here, were purchased from Thermo-Fisher Scientific (Calgary, AB, Canada) or Sigma-Aldrich, Canada Ltd (Oakville, ON, Canada). TEMED, 30 % acrylamide/bis solution, ammonium persulfate, precision plus protein western C standards, precision plus strep-tactin-HRP conjugate were purchased from Bio-Rad Laboratories, Ltd. (Mississauga, ON, Canada) and percoll from GE Healthcare (Uppsala, Sweden). Anti- α -tubulin antibody (mouse monoclonal: clone DM1A), goat anti-mouse IgG (polyclonal), EGF-stimulated A431 cell lysate (phosphotyrosine positive control) and the immobililine-P PVDF transfer membrane (pore size 0.45 μ m) were purchased from Millipore Canada Ltd, (Etobicoke, ON, Canada).

All procedures met the requirements of the University of Saskatchewan ethics and animal care requirements (Animal Use Protocol number: 20140082). Freshly ejaculated semen from eight Holstein bulls of varying fertility from Semex (Guelph, ON, Canada) were used for this experiment. Bull fertility was assessed based on the BFI (Bull Fertility Index) value which is an internal Semex calculated value combining their published 56-day non-return rate (NRR), sire conception rate (SCR) and fertility ratings from Agri-Tech Analytics (ATA). A BFI of 98-101 is average, and those bulls whose BFI lie outside this range are considered to have significantly higher or lower fertility than average ($101 > \text{BFI} < 98$, respectively). The BFI of the eight animals used in this experiment were 106, 102, 99, 98 (two animals), 92, 85 and 78. After collection, each

ejaculate was diluted with egg yolk free 'Clear extender' (proprietary composition, Semex) to a concentration of 60 Million spermatozoa/ml and transported at 18°C to the laboratory in Saskatoon, Saskatchewan, Canada by air within 24 hours.

The various media used in the capacitation assay were prepared as previously described (Thundathil et al., 2006). Modified Tyrode's bicarbonate-buffered medium (Sp-TALPH) was used for washing and modified Tyrode's Hepes buffered medium (Sp-TALP) for culturing the cells. The stock solution of P4 (20 mM) was prepared in absolute ethanol and stored at -20°C while working solutions (50 µM) were prepared in Sp-TALP on the day of the experiment. The ouabain was freshly prepared in Sp-TALP (20mM stock and 50µM working solution). Since both ouabain and P4 were light sensitive, these chemicals were prepared and stored in the dark.

For the capacitation assay, first, the extender was removed by centrifugation (800 × g, 10 min, 25°C) and the pellet was resuspended with Sp-TALPH (1:5, vol:vol). The diluted sperm after density gradient percoll centrifugation (45 % - 90 % percoll gradient; 1:1:1, vol:vol vol; 700 × g, 25 min, 25°C) were washed with Sp-TALPH followed by Sp-TALP (380 x g, 30 min, 25°C) and the final sperm concentration was adjusted to 5×10^6 sperm in a final volume of 100 µl Sp-TALP. Identical aliquots were incubated (39°C, 5 % CO₂, high humidity, 5 hours, dark) under conditions of control (Sp-TALP alone) or with ouabain (Ou) or P4, each at 25, 50 and 100 µM final concentration or combined ouabain plus P4 (Ou+P4; 12.5+12.5, 25+25; 50+50 µM final for each steroid). Samples were taken at 0, 3 and 5 hours of incubation to assess Tyr-p of sperm proteins as a means of capacitation. For this, proteins were extracted from the incubated sperm, snap frozen in liquid nitrogen and stored at -80°C overnight for western immunoblotting (WB) on the next day. The WB was carried out as before (Thundathil et al., 2006; Hickey and Buhr, 2012). Each positive band was analyzed for its molecular weight and volume (sum of the pixel intensity for all pixels

in a given band) using Image Quant TL software (Ver 8.1; GE Healthcare Bio- Science Inc., Baie d'Urfe, QC, Canada). Amount of each MW band was corrected for gel to gel variation based on intensity of the positive control bands.

The data from bulls having a BFI of 98 and above were grouped as good fertility bulls ($n = 5$), and those with fertility indices of 92 and below (92, 85 and 78) were grouped as low fertility bulls ($n = 3$). The effect of different treatments on inducing capacitation was assessed separately in both groups in the same way. Total Tyr-p induced by the treatments was calculated as the total pixel intensity of capacitation sensitive Tyr-p protein bands at each time point and was corrected for time-dependent non-specific phosphorylation by subtracting the total Tyr-p induced by the control sperm at that time. Band volume data were analyzed as logarithmic transformed normalized data in both groups. Impact and interaction of ouabain and P4 were analyzed as randomized complete block design (RCBD) with repeated measures. The covariance model with lowest AIC or BIC values was selected for the repeated measure analysis (Littell et al., 1996). Similarly, the contrast in Tyr-p induced by ouabain to the P4-induced Tyr-p at each time point was calculated by subtracting the Tyr-p induced by P4 from the same doses of ouabain at each time point and analyzed as RCBD with repeated measures. Significant differences and trends were reported at $P < 0.05$ and $0.05 < P < 0.10$, respectively.

5.3 Results and Discussion

There were 15 immunoreactive bands of Tyr-p proteins (214 ± 5.8 , 182 ± 11.3 , 104 ± 10.2 , 81 ± 4.7 , 58 ± 3.0 , 51 ± 0.8 , 47 ± 1.5 , 40 ± 1.0 , 36 ± 0.8 , 31 ± 0.7 , 28 ± 0.7 , 26 ± 0.7 , 24 ± 0.9 , 22 ± 0.6 and 17 ± 1.0 kDa Mean \pm SEM). Analysis of the amount (volume) of each band for each treatment at each incubation time found seven bands to significantly increase in intensity during

capacitating incubation (104 ± 10.2 , 81 ± 4.7 , 58 ± 3.0 , 47 ± 1.5 , 31 ± 0.7 , 28 ± 0.7 , and 17 ± 1.0 kDa; $P < 0.05$), so these seven bands were therefore considered the capacitation-sensitive bands.

In sperm from good fertility bulls ($\text{BFI} \geq 98$, $n = 5$), ouabain overall (all doses pooled) induced a significant increase in total Tyr-p after both 3 and 5 hours, while sperm from low fertility bulls only increased Tyr-p after being exposed to ouabain for 5 hours (Fig. 5.1; Appendix 3.1). Ouabain is a steroid hormone that binds to its receptor Na/K-ATPase to induce signalling through phosphorylating tyrosine proteins and ultimately inducing capacitation (Thundathil et al., 2006), so these fertility-associated differences could be due to differences in the receptor for ouabain or the signalling pathway. Na/K-ATPase binds ouabain with high specificity and is the receptor molecule. It consists of paired α and β subunits (Liu and Aksari, 2006), with four known α isoforms and three β isoforms ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$; $\beta 1$, $\beta 2$, $\beta 3$). Ouabain binds to the α to initiate signalling. Ouabain's binding affinity for $\alpha 3$ significantly exceeds that for $\alpha 1$ or $\alpha 2$ isoforms, so $\alpha 3$ needs less ouabain to activate signalling (Pierre et al., 2008). The slower ouabain-stimulated increase in Tyr-p in low fertility sperm could, therefore, be due to their having a different proportion of the various ATPase α isoforms, thereby changing the speed and extent of ouabain's binding to form an active hormone-receptor complex. Different α isoforms could also alter the signal transduction pathways, because ouabain binding to $\alpha 1$ is associated with Src kinase and pathways that are directly or indirectly mediated through Src, but ouabain binding to either $\alpha 2$ or $\alpha 3$ does not activate Src-dependent signal transduction (Xie et al., 2015).

Bulls of differing fertility also responded differently to P4. Progesterone overall did not induce an increase in total Tyr-p proteins at any time in good fertility bulls but did increase Tyr-p after 5 hours in low-fertility bulls ($P < 0.05$, Fig. 5.1; Appendix 3.1). This is further emphasised by ouabain's induction of significantly more Tyr-p than did P4 at either incubation time in sperm

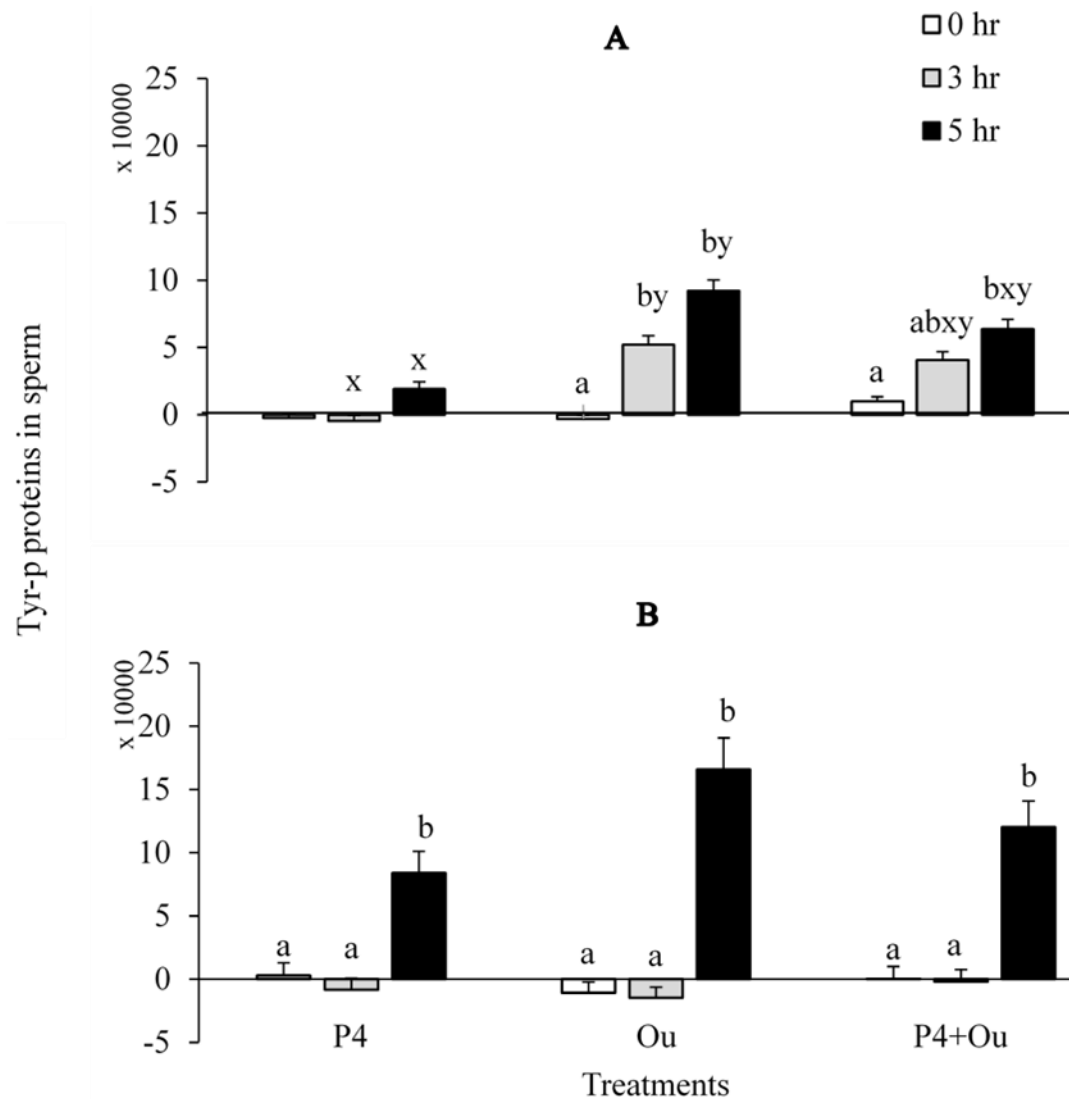


Figure 5.1: Induced tyrosine phosphorylation in sperm from bulls of (A) good and (B) low fertility. Sperm (5×10^6 sperm from $n = 5$ and $n = 3$ ejaculates from good and low fertility bulls, respectively) were incubated in 100 μ l Sp-TALP containing 0 (control), 25, 50 or 100 μ M Ouabain (Ou) or P4 or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hours sperm proteins were extracted for western blot detection of tyrosine phosphorylated (Tyr-p) proteins; amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p bands using Image-Quant analysis after setting the control to zero at each time point. a-b Times without a common letter differ within a treatment, $P < 0.05$. x-y at 3 and 5 hours, treatments without a common letter differ, $P < 0.05$. Treatments at 0 hours did not differ; superscripts are omitted for ease of viewing.

from good fertility bulls, while in low fertility bulls, P4 induced significantly more Tyr-p than ouabain at 3 hr (Fig. 5.2). Progesterone acts through the low-affinity ouabain binding site on the

$\alpha 1$ subunit of Na/K-ATPase in amphibian oocytes (Morrill et al., 2005; 2008) and we have shown that P4 inhibits ouabain response in fresh bull sperm (Chapter 4). The binding characteristics and affinity of P4 for various Na/K-ATPase α isoforms are unknown, but certainly it is reasonable to postulate that P4, like ouabain, would have differing affinities for the different α isoforms. Then differences in the relative proportions of α isoforms present, or structural changes within key isoforms, could alter P4 and ouabain binding with their receptor and impact capacitation and bull fertility.

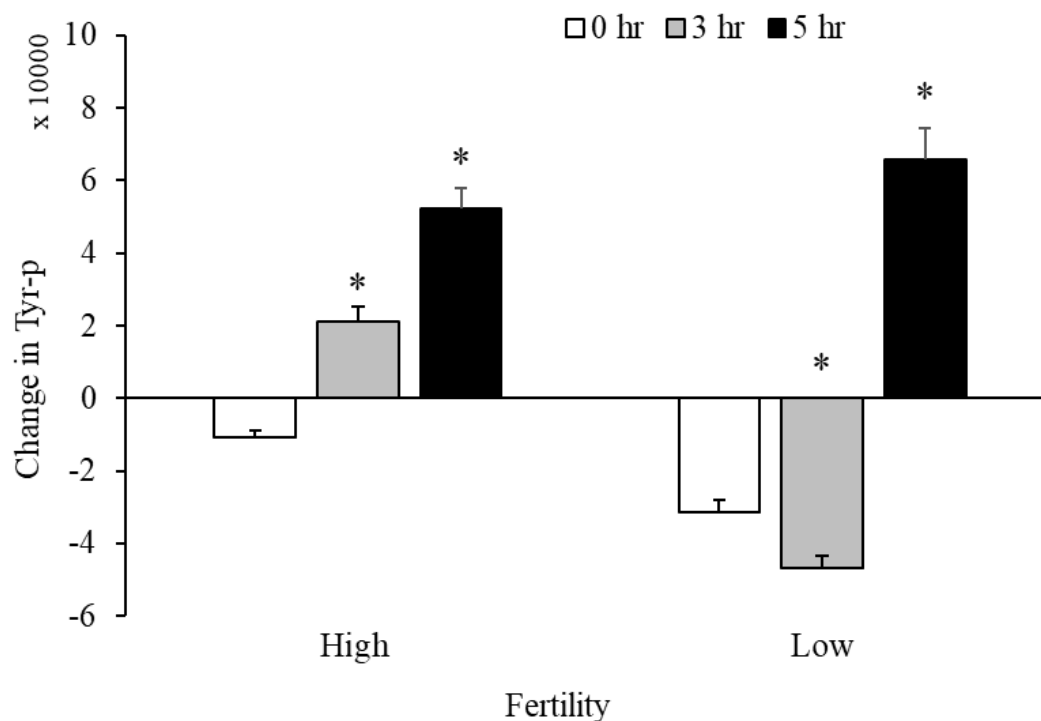


Figure 5.2: Ouabain-induced change in tyrosine-phosphorylation relative to progesterone. Sperm (5×10^6 sperm from $n = 5$ and $n = 3$ ejaculates from good and low fertility bulls, respectively) were incubated in 100 μ l Sp-TALP containing 0 (control), 25, 50 or 100 μ M ouabain or progesterone (P4). At 0, 3 and 5 hr, sperm proteins were extracted for western blot detection of tyrosine phosphorylated (tyr-p) proteins; amount of tyr-p protein was calculated as the total pixel intensity of tyr-p bands using Image-Quant analysis after setting the control to zero at each time point. To compare ouabain to P4, tyr-p induced by P4 was subtracted from the ouabain tyr-p at each time point and the resultant value tested for its difference from zero. Bars significantly > 0 indicate ouabain induced more tyr-p than P4; bars significantly < 0 indicate P4 stimulated more tyr-p than ouabain. *: significantly different from zero, $P < 0.05$

Interestingly, when P4 binds to the low-affinity ouabain-binding site on the α subunit of Na/K-ATPase in amphibian oocytes, it induces only some of ouabain's signalling pathway (Morrill et al., 2005; 2008; 2010). Providing oocytes with both ouabain and P4 led to the steroids competing for the same binding site and P4, which has a higher affinity than ouabain for the low-affinity ouabain binding site on Na/K-ATPase's $\alpha 1$ subunit, displaces ouabain from that low affinity site and induces signalling (Secombe et al., 1989; Morrill et al., 2008). In good fertility bulls, ouabain+P4 (overall) induced an intermediate increase in Tyr-p after 3 and 5 hours of incubation ($P < 0.05$) compared to either steroid alone, while in low fertility bulls Tyr-p induced by ouabain+P4 did not differ from either steroid alone ($P > 0.05$, Fig. 5.1; Appendix 3.1). Specifically, where ouabain alone stimulated a significant increase in Tyr-p both after 3 and 5 hours of incubation of good-fertility sperm, the same amount of ouabain in the presence of P4 induced less Tyr-p, indicating that P4 partially inhibits ouabain action. This P4 suppression of ouabain action is consistent with competition for the binding site and since P4 is less efficient in inducing Tyr-p, it leads to an overall reduced effect.

P4 inhibited ouabain only in sperm from good fertility bulls (Fig. 5.1). The differing efficacy of P4-ouabain interaction in inducing Tyr-p in sperm from good and low fertility bulls could be due to the difference in the amount/distribution of specific isoforms with specific affinities for ouabain/P4, or an altered structure of key isoforms affecting ouabain/P4 binding, or differences in the signalling pathways activated. In the present study, spermatozoa from good fertility animals were more sensitive to ouabain, resulting in an early induction of Tyr-p of sperm proteins whereas, in low fertility animals, ouabain induced a late response. Furthermore, P4 stimulated Tyr-p in low- but not good-fertility animals. This clearly indicates that the isoforms

which have the higher affinity for ouabain, or who have ouabain-sensitive signalling pathways, are more concentrated on sperm from good fertility bulls than from low fertility bulls. Also, the response of low-fertility sperm to P4 suggests that the isoforms concentrated on the low fertility sperm have more affinity for P4 than the isoforms on good fertility bull sperm. Certainly, multiple α isoforms exist in the head plasma membrane of bull sperm where the capacitation-associated signals begin (Hickey and Buhr, 2012). All the isoforms also exist on the rafts, or signalling platforms, found in the head plasma membrane of sperm, confirming their potential involvement in signalling events (Chapter 3). The existence of variation in the amount, distribution, location or structure of various isoforms of Na/K-ATPase on sperm from bulls of varying fertility is as yet unknown, but these results suggest this knowledge could be most useful. However, this experiment used data from only 8 animals to study the fertility related differences in the action of ouabain and P4 and a larger sample size is required to definitively identify the fertility difference. Thus, future studies using ejaculates from large population of bulls are recommended to study the fertility related differences in the action of ouabain and P4.

In conclusion, the *in vitro* capacitation inducing agents ouabain and P4 act differently on spermatozoa from bulls of good or low *in vivo* fertility, and good fertility sperm are more sensitive to the major physiological capacitation-inducer ouabain.

6.0 GENERAL DISCUSSION

This research conclusively demonstrates for the first time the existence of signalling platform ‘rafts’ on the HPM of bovine sperm and the presence of Na/K-ATPase isoforms $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$ and $\beta 3$ on these raft domains, supporting the potential involvement of these isoforms on capacitation-associated signalling. The presence of the $\alpha 2$ isoform has been uniquely here verified in bovine sperm HPM. This study also confirms for the first time the existence of an interaction between the two steroid hormones ouabain and P4 on inducing bull sperm capacitation, and suggests that while ouabain is the primary hormone, both P4 and ouabain can act through binding to Na/K-ATPase to induce signaling associated with bull sperm capacitation.

6.1 Na/K-ATPase Isoforms on the Raft and Non-Raft Fractions of HPM

Even though it is known that ouabain acts through Na/K-ATPase to induce capacitation of bull sperm, the exact isoforms involved have not been characterized so far. All the Na/K-ATPase isoforms are here shown to be present in the bull sperm HPM where the capacitation-associated signals begin. Since rafts are signalling platforms on the cell membrane and the involvement of rafts has been confirmed in capacitation, those isoforms on the raft would have a higher probability of involvement in capacitation-associated signalling. These results demonstrate the existence of signalling platform raft on bull sperm HPM and for the first-time isolated these lipid rafts as DRMs (Chapter 3). Western blotting of proteins extracted from the raft fractions confirmed the presence of ATPase isoforms $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$ and $\beta 3$ in the HPM raft fractions. Even though all the isoforms were present in the raft, and indeed in the non-raft portion of the HPM, each isoform’s profile had characteristics uniquely associated with the raft, and concentrations were suggestive of a signalling pool of ATPase on HPM raft.

Among the various subunit isoforms, $\alpha 3$ and $\beta 1$ constituted the major portion of Na/K-ATPase in the HPM as well as on its raft fractions. Ouabain binds to the binding sites on the α subunit of Na/K-ATPase to induce signalling (Kaplan, 2002). For the Na/K-ATPase to transduce signalling, the α subunit should be paired with the β (Liu and Askari, 2006). Signalling pathways induced by ouabain-ATPase interaction is isoform specific (Pierre et al. 2008). The $\alpha 1$ acts through tyrosine kinase Src that subsequently activates different signalling pathways including the Ras/Raf/ERK while $\alpha 3$ acts through Src-independent pathways involving PI3K and PKC in mammalian cell lines (Tian et al., 2006; Pierre et al. 2008; Xian et al, 2015; Madan et al. 2016). Presence of downstream signalling molecules of both $\alpha 1$ and $\alpha 3$ specific signalling pathways involving cAMP/PKA, Src/RTK/ PLC/IP3/DAG/PKC and MAPK have also been reported in bull sperm on incubation with ouabain (Daniel et al. 2010; Newton et al., 2010; Rajamanickam et al., 2017) which further confirms the involvement of these isoforms on ouabain induced signalling in bull sperm. Even though the signalling properties of $\alpha 2$ have not been confirmed yet in somatic cells (Pierre et al. 2008; Xie et al. 2015), the presence of $\alpha 2$ in the raft fraction of HPM warrants further evaluation of its possible role in signalling in germ cells.

The binding of ouabain to the α subunit depends on the affinity of these isoforms for ouabain which significantly varies among isoforms. Among different α isoforms, the $\alpha 3$ is reported to have higher affinity for ouabain than $\alpha 1$ and so significantly less ouabain is needed to bind and activate signalling through $\alpha 3$ relative to $\alpha 1$ in Sf-9 insect cells (Pierre et al. 2008) and in mammalian cells (O'Brien et al., 1993). The higher amount of $\alpha 3$ relative to other α isoforms in the raft fractions of bull sperm HPM and the higher affinity of $\alpha 3$ for ouabain likely indicates that $\alpha 3$ along with $\beta 1$ plays a major role in ouabain induced signal transduction for inducing capacitation in bull sperm.

Results of these experiments (Chapter 3) showed that the Na/K-ATPase isoforms were not restricted to the raft fraction of bull sperm HPM, instead, all the isoforms were also present in the non-raft fractions of HPM. Even though isoforms on a raft could be highly likely to be involved in signalling, non-raft isoforms cannot be eliminated from potential involvement in signalling. A recent study on bull sperm reported the involvement of Na/K-ATPase isoforms from both the raft and non-raft fractions obtained from homogenates of whole bull sperm in capacitation-associated signalling induced by ouabain (Rajamanickam et al., 2017). These findings together with the detection of Na/K-ATPase subunit isoforms in the non-raft fractions of bull sperm HPM in the present study suggests the presence of a signalling pool of Na/K-ATPase on the non-raft.

Even though this study (Chapter 3) confirms the existence of Na/K-ATPase isoforms in both raft and non-raft fractions of HPM, the total amount of isoform protein was significantly less in the combined fractions than in HPM (Fig. 3.10). The exact reason for this loss is not known, but it could be related to the protein loss in the discarded ultracentrifugation pellet, or during concentration using centrifugal filters. Thus, it would be worth studying the pellet composition to evaluate any ATPase lost through the discarded pellet.

In these experiments, the protein bands were detected with chemiluminescence system and quantified using Image Quant software. Since western blots and protein detection by chemiluminescence system have the limitation of having non-linearity in color (increases in protein level will not produce a proportional increase in signal intensity), we used an optimum antibody concentration and protein load for each isoform so that the positive bands gave the optimum visible band intensity and were not oversaturated. Also, gel to gel variation in the intensity of bands were corrected using inter-gel control. Furthermore, this experiment used same

amount of antibody to detect positive bands on each fraction for each isoform so that comparing band volumes between fractions are valid. However, future studies using fluorescence detection system could provide the advantage of improved stability of the signal over chemiluminescence, increased sensitivity and a broader dynamic range would be recommended to get more consistent and quantitative measurements.

In the current study, Na/K-ATPase isoforms were studied in raft and non-raft fractions obtained from the HPM of fresh uncapacitated sperm (Chapter 3). The amount/distribution of various Na/K-ATPase isoforms may vary with the capacitation status of sperm. In somatic cells, initiation of signalling is associated with the recruitment of various membrane receptor-like EGFR as well as other signaling molecules in and out from the raft domain mediated by the cytoskeleton (Goswami et al. 2008; Head et al. 2014; Lozano et al., 2016). However, the movement of Na/K-ATPase during signalling induction has not been studied much in any cell type. Capacitation is also associated with coalescence of raft towards the apical plasma membrane (Khalil et al., 2006). Aggregation of rafts permits the assembly of various signalling complexes into a new environment and activation of a new interaction between molecules which also facilitates the change in raft structure. So, it would be worthwhile to study the distribution of Na/K-ATPase in the HPM of capacitating sperm to study the changes in the distribution of Na/K-ATPase associated with capacitation.

6.2 Induction of Tyr-p by Ouabain and P4

Ouabain significantly phosphorylated seven sperm protein bands (104, 81, 58, 47, 31, 28, and 17 kDa; Chapter 4) and induced capacitation of bull sperm. Ouabain binds to the α subunit of Na/K-ATPase which has two ouabain binding sites, a low-affinity ouabain binding site located between TM1 and TM2 and a high affinity binding site located between TM4 and TM6 (Morrill

et al., 2008) to induce signalling. Progesterone is another steroid hormone which can induce capacitation of equine and human sperm but shows contradictory responses in bull sperm, being reported to either only induce capacitation, or only stimulate the AR in previously-capacitated sperm, or to induce both capacitation and the AR (Therien and Manjunath, 2003; Lukoseviciute et al., 2004; 2005). Progesterone induced Tyr-p of three sperm proteins (104, 47 and 17 kDa; Chapter 4), but did not induce capacitation of bull sperm. There was an interaction between ouabain and P4 on capacitation, specifically P4 partially inhibited ouabain-induced Tyr-p of bull sperm. Progesterone acts through mPR to exert its action on sperm, but the exact nature of this receptor molecule in bull sperm is not known. In amphibian oocytes, Na/K-ATPase acts as a functional receptor for P4, where P4 binds to the low affinity ouabain binding site on Na/K-ATPase to exert its action. In oocytes, P4 binding to the low-affinity ouabain binding site on Na/K-ATPase induced only some of the signalling cascades that ouabain did, stimulating DAG and MAPK, but not cAMP/PKA (Morrill et al. 2010). This suggests the possibility that both ouabain and P4 can act through Na/K-ATPase to phosphorylate some of the same proteins, but ouabain is more efficient as ouabain can activate more signalling pathways and induce more tyrosine phosphorylation. Results of present studies (Chapter 4) support the existence of these signaling pathways on bull sperm where P4 phosphorylated three proteins while ouabain phosphorylated the same three proteins along with four other proteins indicating the possibility that P4 activates the same kinases as does ouabain, but ouabain activates more kinases and was more effective in inducing Tyr-p of sperm proteins. These results in bull sperm along with the findings on amphibian oocytes support our hypothesis that in bull sperm ouabain is the primary hormone inducing capacitation, while P4 acts as a less-effective ouabain agonist through Na/K-ATPase to induce Tyr-p of sperm proteins. Moreover, when ouabain and P4 are both present, P4 competes and can partially inhibit ouabain-induced Tyr-p in bull sperm. In amphibian oocytes and in the canine kidney, P4 can replace

ouabain from its low-affinity ouabain binding site on the α subunit of Na/K-ATPase (Seccombe et al., 1989; Morrill et al., 2008). This same mechanism could exist in bull sperm, allowing the inhibition of ouabain induced Tyr-p by P4. Since P4 is less effective in inducing Tyr-p in bull sperm, the overall response is reduced. This finding further reinforces the hypothesis that ouabain is the primary stimulator of capacitation, and P4's ability to induce bull sperm capacitation is by mimicking ouabain at the same binding site on Na/K-ATPase.

It is important to note that the binding of P4 to the low-affinity ouabain binding sites on Na/K-ATPase has only been studied in the $\alpha 1$ subunit (Morrill et al., 2008; Khalid et al., 2014), and no reports are available on ouabain binding sites (low and high affinity) on other α isoforms. Since $\alpha 3$ is here implicated as the possible major α isoform on HPM and possible candidate for ouabain action on bull sperm, inhibition of P4 on ouabain induced Tyr-p suggests the possibility of existence of low and high affinity ouabain binding sites on other alpha isoforms as well.

Importantly, the response of bull sperm to ouabain/P4 induced capacitation varies with fertility. Sperm from good-fertility bulls were more sensitive to ouabain and less sensitive to P4 than sperm from low-fertility bulls (Chapter 5). Since it is established that ouabain and possibly P4 act through the same binding site on Na/K-ATPase to induce signalling for bull sperm capacitation, the variation in the response of ouabain and P4 to bull sperm of varying fertility clearly suggests that the isoforms through which ouabain and P4 act are differently distributed in good and low fertility bull sperm. Therefore, it would be useful to study the distribution of various Na/K-ATPase on bull sperm of varying fertility as potential indices for bull fertility.

Since Na/K-ATPase-ouabain interaction is critical for inducing capacitation, Na/K-ATPase should impact bull fertility. Therefore, identifying this protein, and intracellular signalling pathways in bull sperm of varying fertility could improve understanding of the mechanism for

capacitation and the fertility of bulls. This could improve bull selection and also to deliver bull semen of known fertility. Predicting the fertility of young bulls would help producers eliminate sub/low fertility bulls early and reduce the costs associated with housing and maintenance of these bulls. The use of semen with known fertility for insemination will reduce the inter-calving interval and number of open cows and increase productivity and profitability. Overall this knowledge will help us to protect, predict and improve bull sperm fertility which would revolutionize the areas of artificial insemination and animal agriculture.

7.0 GENERAL CONCLUSION

This study supported the hypothesis that the signalling platform rafts typically associated with signalling exist on bull sperm HPM. All the Na/K-ATPase subunit isoforms $\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$, and $\beta 3$ are present on these signalling domains, with $\alpha 2$ being identified for the first time in bovine sperm HPM. Contrary to our hypothesis that Na/K-ATPase subunit isoforms which are involved in capacitation-associated signalling preferentially localize in the signalling platform raft, the results here demonstrated for the first time that none of the Na/K-ATPase isoforms are restricted to raft, instead all the isoforms are present both in the raft and non-raft fraction of HPM. Yet, the unique profile and concentration of these isoforms on the raft fractions are consistent with the possible involvement of these isoforms on capacitation-associated signalling. Among these isoforms, $\alpha 3$ and $\beta 1$ are the predominant Na/K-ATPase isoforms in the raft fractions of bull sperm HPM which suggests that these are likely candidate isoforms for ouabain induced signalling stimulating capacitation in bull sperm.

The results support the hypothesis that ouabain and P4 can interact in bull sperm capacitation. Ouabain increases Tyr-p of seven sperm protein bands and induces capacitation of bull sperm. Progesterone phosphorylates three of the same proteins as ouabain but cannot effectively induce capacitation of bull sperm. Furthermore, the results here also imply that ouabain and possibly P4 act through the same binding site on Na/K-ATPase and induce Tyr-p of same sperm proteins. This hypothesis is further strengthened by P4's partial inhibition of the sperm response to ouabain, which could be due to P4 competing with ouabain for a binding site on Na/K-ATPase (possibly the low-affinity binding site as in amphibian oocytes). Since P4 is less efficient at inducing Tyr-p of sperm proteins, there is an overall decrease in Tyr-p and capacitation. Overall, these results confirmed that ouabain is more effective at inducing Tyr-p of sperm proteins, and

therefore possibly is the primary, physiological inducer of bovine capacitation. The results also detected a fertility-related difference in the action of ouabain and P4 on bull sperm where ouabain was found to be more effective in good fertility animals while P4 induces its effect, although small, only in low fertility animals. These differences in response of bull sperm to ouabain and P4 suggests fertility related differences in the amount/distribution of various Na/K-ATPase isoforms or signal response elements in bull sperm through which ouabain and P4 act to affect sperm. Therefore, a comprehensive investigation of sperm from good and low fertility bulls to determine the amount/distribution of ATPase would be a great tool to establish the relationship between ATPase isoforms and fertility.

Fertility is an important trait in both the dairy and beef cattle industries and significantly impacts productivity and profitability. Capacitation is one of the major molecular events that make the sperm structurally and functionally competent for fertilization. The findings here provide a better understanding about the major molecular mechanisms associated with capacitation and the biological molecules closely associated with it, providing insights for developing methodology for predicting bull sperm fertility at younger reproductive stages.

Current research opens the possibility of new researches to address questions unanswered in these studies. Distribution of Na/K-ATPase used HPM from non-capacitated sperm from bulls of mixed fertility. Future research studying the distribution of Na/K-ATPase isoforms on non-capacitated and capacitated sperm could identify precise mechanisms involved in fertility regulation by Na/K-ATPase. Also, this study identified seven capacitation related Ty-P protein bands are associated with successful capacitation. Detailed proteomic studies on these proteins to identify the exact role of these proteins on induction of capacitation would be useful for providing insights to the underlying mechanistic pathways.

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APPENDICES

APPENDIX 1

Appendix 1.1: Profile of Na/K-ATPase $\alpha 2$ in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 5 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase $\alpha 2$ antibody.

MW (kDa)	Amount				Percentage ^x			
	HPM	Non-Raft	Raft	SEM ^y	HPM	Non-Raft	Raft	SEM ^y
129 \pm 2.3	492574 ^a	139455 ^b	173269 ^b	113368	4.6 ^a	2.9 ^a	14.9 ^b	3.4
77 \pm 1.2	2874818 ^a	742368 ^b	- ^z	527395	26.8	13.5	-	6.7
54 \pm 0.9	777625	-	398634	225930	7.4 ^a	-	36.0 ^b	9.5
42 \pm 0.7	619520	721581	-	228922	5.8 ^a	23.5 ^b	-	5.6
34 \pm 0.6	1317702	2114862	-	692318	12.2 ^a	44.7 ^b	-	12.2
30 \pm 0.4	4308929	-	-	724436	40.1	-	-	10.7
25 \pm 0.7	317775*	497674	706586*	175244	3.0 ^a	16.2 ^b	62.5 ^c	11

^x Calculated as percentage of individual band volume over total volume for each fraction.

^y Pooled standard error of mean.

^z No band detected.

a-c means without a common letter differ among fractions (P < 0.05), using contrast analysis of log transformed normalized data.

* the two values tend to differ (0.1 > P > 0.05).

Appendix 1.2: Profile of Na/K-ATPase $\alpha 1$ in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 5 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase $\alpha 1$ antibody.

MW (kDa)	Amount				Percentage ^X			
	HPM	Non-Raft	Raft	SEM ^Y	HPM	Non-Raft	Raft	SEM ^Y
158 \pm 2.0	673336	- ^Z	-	306570	16	-	-	4.7
131 \pm 2.8	127631	-	-	100663	1.2	-	-	0.6
110 \pm 2.4	157803 ^a	429338 ^b	183304 ^a	130381	4.2 ^a	33.4 ^b	22.2 ^b	12.1
96 \pm 1.4	155050	-	-	86471	2.9	-	-	1
72 \pm 1.5	641844	436263	296588	272101	17.2 ^a	34.0 ^b	28.1 ^b	13.2
64 \pm 1.4	476775 ^a	446190 ^{ab*}	197738 ^{b*}	284715	13.2	13.5	7	5.6
51 \pm 0.6	244409	-	-	103043	6.6	-	-	1.8
47 \pm 0.8	537616	-	-	346225	5.4	-	-	2.2
43 \pm 0.6	262577	-	-	146439	5.9	-	-	2.1
40 \pm 0.8	296084	559501		339915	3.5	8.5	-	3.2
39 \pm 0.7	439152 ^a	58233 ^b	-	176200	8.1	8.5	-	3.6
34 \pm 0.6	251677	548367	-	318444	5.2 ^a	16.5 ^b	-	6
31 \pm 0.6	230245	636220	429123	354649	5.5 [*]	9.6	12.7 [*]	4.7
29 \pm 0.4	219060	-	-	122170	2.9	-	-	1
25 \pm 0.7	649917 ^a	160348 ^b	893767 ^a	350971	11.3 ^a	2.4 ^b	60.5 ^c	12.6
22 \pm 0.5	180466	-	-	142334	2.2	-	-	1.1

^X Calculated as percentage of individual band volume over total volume for each fraction.

^Y Pooled standard error of mean.

^Z No band detected.

a-c means without a common letter differ among fractions ($P < 0.05$), using contrast analysis of log transformed normalized data.

* the two values tend to differ ($0.1 > P > 0.05$).

Appendix 1.3: Profile of Na/K-ATPase $\alpha 3$ in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 5 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase $\alpha 3$ antibody.

MW (kDa)	Amount				Percentage ^X			
	HPM	Non-Raft	Raft	SEM ^Y	HPM	Non-Raft	Raft	SEM ^Y
154 \pm 4.0	13207561	- ^Z	-	4556608	8.1	-	-	2.17
114 \pm 2.8	12206754 ^a	1306419 ^b	463796 ^c	1905341	7.1	10.6	7.4	2.17
90 \pm 1.9	15059205	-	-	4028337	8.8	-	-	1.81
72 \pm 2.3	6775339	-	-	2861903	4.6	-	-	1.52
68 \pm 1.6	17454359 ^a	1953826 ^b	971018 ^b	2723549	10.2	15.4	8.6	2.72
55 \pm 1.3	17771383 ^a	1783164 ^b	1802527 ^b	2790673	10.4	13.7	13.5	3.57
49 \pm 1.1	15005089	-	-	4488022	7.7	-	-	1.77
44 \pm 0.9	11939944	-	-	3193935	7.0	-	-	1.43
40 \pm 0.9	23603369 ^a	1992100 ^b	-	4490943	13.8	8.3	-	2.78
32 \pm 0.4	37920408	-	-	6548303	19.4	-	-	4.47
27 \pm 0.6	7346981	-	-	2197482	3.8	-	-	0.87
25 \pm 0.6	9127478	6458451	6709265	2251786	4.7 ^a	63.9 ^b	83.9 ^b	14.06
23 \pm 0.7	3019495	-	-	1274529	1.6	-	-	0.54
21 \pm 0.6	2423686	-	-	1023038	1.3	-	-	0.43

^X Calculated as percentage of individual band volume over total volume for each fraction.

^Y Pooled standard error of mean.

^Z No band detected.

a-c means without a common letter differ among fractions (P < 0.05), using contrast analysis of log transformed normalized data.

Appendix 1.4: Profile of Na/K-ATPase β 1 in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 7 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase β 1 antibody.

MW (kDa)	Amount				Percentage ^X			
	HPM	Non-Raft	Raft	SEM ^Y	HPM	Non-Raft	Raft	SEM ^Y
103 \pm 1.84	985594	2186472	952362	577553	0.9 ^a	5.2 ^b	5.5 ^b	1.3
71 \pm 1.18	25599926 ^a	2780663 ^b	2227967 ^b	6009799	21.5 ^a	6.7 ^b	13.6 ^{ab}	4.7
56 \pm 0.87	25329999 ^a	11028573 ^b	8086447 ^b	6449138	21.3 ^a	34.1 ^{ab}	59.6 ^b	10.6
50 \pm 1.00	6978189 ^a	- ^Z	887443 ^b	2014949	5.9	-	4.8	1.9
47 \pm 0.83	6647823	-	-	2681067	5.6	-	-	1.8
42 \pm 0.79	17475317 ^a	3422562 ^b	2268660 ^b	4179299	14.7	9.7	8.9	3.7
39 \pm 0.67	20804919 ^a	11711712 ^a	3321078 ^b	5453420	17.5 ^{ab}	33.3 ^a	12.8 ^b	6.6
31 \pm 0.60	1917245	-	-	851065	1.8	-	-	0.6
24 \pm 0.45	3545182 ^a	-	504539 ^b	1122010	3.3	-	2.9	1.0
20 \pm 0.76	2799916	-	-	2351090	4.6	-	-	3.3
17 \pm 0.33	915293	1845203	1250557	582593	0.8 ^a	4.7 ^b	5.6 ^b	1.3

^X Calculated as percentage of individual band volume over total volume for each fraction.

^Y Pooled standard error of mean.

^Z No band detected.

a-c means without a common letter differ among fractions (P < 0.05), using contrast analysis of log transformed normalized data.

Appendix 1.5: Profile of Na/K-ATPase $\beta 2$ in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 6 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase $\beta 2$ antibody.

MW (kDa)	Amount				Percentage ^X			
	HPM	Non-Raft	Raft	SEM ^Y	HPM	Non-Raft	Raft	SEM ^Y
112 \pm 1.1	383387 ^{ab*}	539717 ^a	206241 ^{b*}	143337	3.4 ^a	21.8 ^{b*}	11.0 ^{b*}	4
73 \pm 1.2	4032915 ^a	1514809 ^b	432138 ^c	857017	36.7 ^{ab}	61.3 ^a	20.5 ^b	12
52 \pm 1.0	1013378	- ^Z	-	362829	8.9	-	-	2.4
46 \pm 1.1	735643	-	-	311253	7.2	-	-	2.5
42 \pm 1.5	588305	-	-	199795	5.3	-	-	1.3
40 \pm 1.2	1108258 ^a	386003 ^b		285954	10.1	16.4		3.9
33 \pm 1.2	1095477 ^a	303853 ^b	254435 ^b	284178	10.0	10.2	13.4	4.0
31 \pm 1.1	1206459	-	-	431191	10.6	-	-	2.8
25 \pm 1.0	373249 ^a	-	976471 ^b	251038	3.4 ^a	-	55.9 ^b	10
22 \pm 1.1	638132	-	-	228137	5.6	-	-	1.5

^X Calculated as percentage of individual band volume over total volume for each fraction.

^Y Pooled standard error of mean.

^Z No band detected.

a-c means without a common letter differ among fractions (P < 0.05), using contrast analysis of log transformed normalized data.

* the two values tend to differ (0.1 > P > 0.05).

Appendix 1.6: Profile of Na/K-ATPase $\beta 3$ in the head plasma membrane and its raft and non-raft fractions. Molecular weight (MW; mean kDa \pm SEM); amount (band volume /mg protein, mean \pm SEM obtained from image quant analysis after correction for internal standard) and percentage (mean \pm SEM) of individual bands from the head plasma membrane (HPM) isolated from fresh bull sperm (n = 7 ejaculates) and the raft and non-raft fractions obtained from each HPM preparation. Proteins extracted from the three sources were subjected to SDS gel electrophoresis and bands detected by exposure to anti-Na/K-ATPase $\beta 3$ antibody.

MW (kDa)	Amount				Percentage ^x			
	HPM	NonRaft	RAFT	SEM ^y	HPM	NonRaft	RAFT	SEM ^y
114 \pm 0.9	375871 ^{ab}	661391 ^a	183873 ^b	150501	2.8 ^a	31.2 ^b	8.2 ^c	4.9
73 \pm 1.1	3167965 ^a	868741 ^b	49772 ^c	670761	24.1 ^a	32.8 ^a	2.6 ^b	6.7
49 \pm 0.9	1170568	- ^z	-	388512	8.6	-	-	2.3
41 \pm 1.1	674954*	238804*	-	177806	5.0	6.5	-	2.1
40 \pm 1.5	1108369 ^a	234896 ^b	-	386632	6.3	12.4	-	4.8
39 \pm 1.2	1130646 ^a	185313 ^b	-	289493	8.1	12.2	-	3.9
33 \pm 1.2	1509818 ^a	237281 ^b	128849 ^b	296463	11.1	11.5	7.2	3.3
31 \pm 1.2	1189686 ^a	-	69606 ^b	280197	8.8 ^a	-	2.9 ^b	2.2
29 \pm 0.9	1246064	-	-	413569	9.0	-	-	2.5
26 \pm 1.2	837436 ^a	71998 ^b	1375049 ^a	378980	6.2 ^a	1.6 ^b	71.3 ^c	17.1
24 \pm 1.3	879668	-	-	292943	5.0	-	-	17.9
21 \pm 1.0	489383	-	-	186748	3.3	-	-	1.0
17 \pm 1.1	355614	154168	207192	98166	2.6 ^a	4.3 ^{ab}	9.0 ^b	1.9

^x Calculated as percentage of individual band volume over total volume for each fraction.

^y Pooled standard error of mean.

^z No band detected.

a-c means without a common letter differ among fractions (P < 0.05), using contrast analysis of log transformed normalized data.

* the two values tend to differ (0.1 > P > 0.05).

Appendix 1.7: Total volume of Na/K-ATPase isoforms in the head plasma membrane and its raft and non-raft fractions. Total volume of each Na/K-ATPase subunit isoform (as per Appendix 1.1-Appendix 1.6) calculated by multiplying total volume/ μg protein for each fraction \times total protein in each fraction. Comparisons were made by contrast analysis of log transformed normalized data.

Isoform	Raft	Non-Raft	HPM	SEM*
$\alpha 1$	58595 ^{a x!}	1118949 ^{b x!}	10591899 ^{c x!}	2438841
$\alpha 2$	87693 ^{a x!}	3180662 ^{b xy!}	34907072 ^{c xy!}	8685414
$\alpha 3$	300319 ^{a xy!}	6242554 ^{b xy!}	265174856 ^{c z!}	71858870
$\beta 1$	543563 ^{a y!}	15351135 ^{b y!}	174773254 ^{c zx!}	47458935
$\beta 2$	130210 ^{a xy!}	1985537 ^{b x!}	34886134 ^{c xy!}	8640633
$\beta 3$	117536 ^{a xy!}	1711362 ^{b x!}	32255528 ^{c x!}	7396215

* Pooled standard error of mean.

^{a-b} means without a common letter differ among fractions within an isoform ($P < 0.05$).

^{x-z} means without a common letter differ among isoforms within a fraction ($P < 0.05$).

! This experiment used different antibodies at different concentrations for detecting each isoform and the manufacturer's indicate different antibody affinities for its antigen. Those can contribute uncertainties to the results

APPENDIX 2: CAPACITATION ASSAY

Appendix 2.1: Protein tyrosine phosphorylation of bull sperm exposed to ouabain and progesterone and incubated under capacitating conditions. Bull sperm (5×10^6 sperm in 100 μ l from $n = 5$ ejaculates) were incubated with 50 μ M ouabain (Ou) or progesterone (P4) or with a combination of 25 μ M of each (P4+Ou), or no steroid (control) under capacitating conditions. At 0 and 5 hr sperm proteins were extracted and assayed with SDS –PAGE electrophoresis and western blotting for tyrosine phosphorylated (Tyr-p) protein using anti-phosphotyrosine antibody; amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant (mean \pm SEM). Data were log transformed, normalized and statistically compared by randomized complete block design (RCBD); values reported are the total pixel intensity (amount of Tyr-p).

Treatment	0 h	5 h	SEM [†]
Control	307610	258821 ^x	31326
P4_50	295665	349623 ^{xy}	35679
Ou_50	261216 ^a	492493 ^{by}	43441
P4+Ou_25	301578	389673 ^{xy}	38396

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 5 hours, $P < 0.05$. Values at 0 hr did not differ.

Appendix 2.2: Capacitation of bovine sperm exposed to ouabain and progesterone. Bull sperm (5×10^6 sperm in 100 μ l from $n = 5$ ejaculates) were incubated with 50 μ M ouabain (Ou) or progesterone (P4) or with a combination of 25 μ M of each (P4+Ou), or no steroid (control) under capacitating conditions. After 0 or 5 hr of incubation, identical aliquots from each treatment were exposed to 0 or 5 μ g of lyso-phosphatidylcholine (LPC, a known inducer of the acrosome reaction (AR) in capacitated sperm) for 30 min. Sperm were stained with Fluorescein isothiocyanate conjugated pisum sativum agglutinin (FITC-PSA) and scored as having undergone an acrosome reaction microscopically. The % of LPC-induced acrosome-reacted bull sperm was calculated by first subtracting the 0LPC AR sperm from those undergoing an AR in the presence of LPC at each time period. Data were statistically compared by randomized complete block design (RCBD).

Treatment	0 h	5 h	SEM[†]
Control	19	16 ^x	2.8
P4_50	19	18 ^x	2.8
Ou_50	17	36 ^y	2.8
P4+Ou_25	18 ^{a*}	30 ^{b* xy}	3.1

[†] Pooled standard error of mean.

^{a-b}: means without a common letter differ between times, $P < 0.05$.

* Times 0 and 5 hours tend to differ, $P=0.08$.

^{x-y}: at 5 hours, treatments without a common letter differ, $P < 0.05$. There were no significant differences at 0 hr.

Appendix 2.3: Protein tyrosine phosphorylation in bull sperm induced by overall doses of ouabain, progesterone and ouabain+progesterone under capacitating conditions. Bull sperm (5×10^6 sperm from $n = 9$ bulls) were incubated for 5 hours in 100 μ l Sp-TALP containing 0 (control), 25, 50 or 100 μ M ouabain (Ou) or progesterone (P4) or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hr sperm proteins were extracted and assayed for tyrosine phosphorylated (Tyr-p) protein in sperm using anti-phosphotyrosine antibody; amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant (mean \pm SEM). Data within a steroid (Ou, P4, Ou+P4) were pooled, log transformed and normalized, and compared by randomized complete block design (RCBD) with repeated measures. Values reported are the total pixel intensity (amount of Tyr-p).

MW	0 h	3 h	5 h	SEM [†]
Control	68423	110205 ^{xy}	121759 ^{xy}	9011
P4	70486 ^a	111481 ^{bx}	168927 ^{bx}	9556
Ouabain	67298 ^a	146487 ^{by}	254683 ^{by}	13379
Ouabain+P4	83541 ^a	143516 ^{by}	212765 ^{bxy}	12015

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 3 and 5 hours, $P < 0.05$. There were no significant differences at 0 hr.

Appendix 2.4: Increase in protein tyrosine phosphorylation in bull sperm induced by overall doses of ouabain, progesterone and ouabain+progesterone under capacitating conditions.

Bull sperm (5×10^6 sperm from $n = 9$ bulls) were incubated for 5 hours in 100 μ l Sp-TALP containing 0 (control), 25, 50 or 100 μ M ouabain (Ou) or progesterone (P4) or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hr sperm proteins were extracted and assayed for tyrosine phosphorylated (Tyr-p) protein in sperm using anti-phosphotyrosine antibody and the amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant. Actual increase as well as the percentage increase in amount of Tyr-p proteins from 0 hour over 3 and 5 hours were calculated and analyzed (mean \pm SEM). Data within a steroid (Ou, P4, Ou+P4) were pooled, log transformed and normalized, and compared by randomized complete block design (RCBD). Values reported are the actual increase in pixel intensity (increase in Tyr-p) and the percentage increase in pixel intensity (percentage increase in Tyr-p).

MW	3 -0 h	5-0 h	SEM [†]
Control	46437	57841 ^x	10776
P4	37831 ^a	85531 ^{bxz}	9399
Ouabain	80061 ^a	190394 ^{by}	12293
Ouabain+P4	70454 ^a	127307 ^{bz}	10825

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 5 hours, $P < 0.05$. There were no significant differences at 3-0 hr.

Appendix 2.5: Protein tyrosine phosphorylation of bull sperm exposed to various doses of ouabain, progesterone and ouabain+progesterone incubated under capacitating conditions.

Bull sperm (5×10^6 sperm from $n = 9$ bulls) were incubated for 5 hours in 100 μ l Sp-TALP containing 0 (control), 12.5, 25, 50 or 100 μ M ouabain (Ou) or progesterone (P4) or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hr sperm proteins were extracted and assayed for tyrosine phosphorylated (Tyr-p) protein in sperm using anti-phosphotyrosine antibody; amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant (mean \pm SEM). Data were log transformed and normalized and statistically compared by randomized complete block design (RCBD) with repeated measures. Values reported are the total pixel intensity (amount of Tyr-p).

MW	0 h	3 h	5 h	SEM [†]
Control	68423	110205	121759 ^x	8782
P4_12.5	84295	127791	163230 ^{xy}	11113
P4_25	81489	107647	177828 ^{xy}	10911
P4_50	72963	117166	158234 ^{xy}	10271
P4_100	58884 ^a	109850 ^{ab}	171317 ^{bxy}	10247
Oub_12.5	61010 ^a	108218 ^{ab}	203704 ^{by}	11475
Oub_25	54051 ^a	113606 ^a	245358 ^{by}	13160
Oub_50	71269 ^a	142430 ^{ab}	268534 ^{by}	15090
Oub_100	77857 ^a	158016 ^{ab}	250669 ^{by}	14775
P4+Oub_12.5	68565 ^a	151775 ^{ab}	225684 ^{by}	13529
P4+Oub_25	108818 ^a	150072 ^a	202535 ^{bxy}	13526
P4+Oub_50	78127 ^a	129718 ^{ab}	210669 ^{bxy}	12574

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 5 hours, $P < 0.05$. There were no significant differences at 0 or 3 hr.

Appendix 2.6: Increase in protein tyrosine phosphorylation of bull sperm exposed to various doses of ouabain, P4 and ouabain+P4 and incubated under capacitating conditions. Bull sperm (5×10^6 sperm from $n = 9$ bulls) were incubated for 5 hours in 100 μ l Sp-TALP containing 0 (control), 12.5, 25, 50 or 100 μ M Ouabain (Ou) or P4 or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hr sperm proteins were extracted and assayed for Tyr-p protein in sperm using anti-phosphotyrosine antibody and the amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant. Actual increase as well as the percentage increase in amount of Tyr-p proteins from 0 hour over 3 and 5 hours were calculated and analyzed (mean \pm SEM). Data were log transformed and normalized and statistically compared by randomized complete block design (RCBD). Values reported are the actual increase in pixel intensity (increase in Tyr-p) and the percentage increase in pixel intensity (percentage increase in Tyr-p).

Increase in Tyrosine phosphorylation				Percentage increase in Tyrosine phosphorylation		
MW	3-0 h	5-0 h	SEM [†]	3-0 h	5-0 h	SEM [†]
Control	46437	58406 ^x	10773	58	70 ^x	16
P4_12.5	43178	88499 ^{xy}	11809	43	101 ^{xy}	17
P4_25	26717	82969 ^{xy}	10786	26	117 ^{xy}	17
P4_50	37935	75607 ^{xy}	10832	56	114 ^{xy}	18
P4_100	51548	98384 ^{xy}	11791	79	186 ^{xy}	24
Oub_12.5	58180	150032 ^{xy}	13054	72	231 ^{xy}	27
Oub_25	80061	182991 ^y	14435	100	347 ^y	36
Oub_50	78711 ^a	208644 ^{by}	14927	94	336 ^y	35
Oub_100	82037	180104 ^{xy}	14255	100	221 ^{xy}	27
P4+Oub_12.5	80554	156776 ^{xy}	13644	118	227 ^{xy}	28
P4+Oub_25	71524	85971 ^{xy}	12143	35	84 ^{xy}	16
P4+Oub_50	60165	143801 ^{xy}	12934	62	167 ^{xy}	22

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 5 hours, $P < 0.05$. There were no significant differences at 3-0 hr.

APPENDIX 3

Appendix 3.1: Protein tyrosine phosphorylation in sperm from bulls of good and low fertility induced by overall doses of ouabain, progesterone and ouabain+progesterone under capacitating conditions. Bull sperm (5×10^6 sperm from $n = 5$ and $n = 3$ ejaculates from good and low fertility bulls, respectively) were incubated for 5 hours in 100 μ l Sp-TALP containing 0 (control), 25, 50 or 100 μ M ouabain (Ou) or progesterone (P4) or combined P4+Ou (each at 12.5, 25 & 50 μ M). At 0, 3 and 5 hr sperm proteins were extracted and assayed for tyrosine phosphorylated (Tyr-p) protein in sperm using anti-phosphotyrosine antibody; amount of Tyr-p protein was calculated as the total pixel intensity of Tyr-p protein bands which increased in intensity over time using Image-Quant after setting the control to zero at each time point (mean \pm SEM). Data within a steroid (Ou, P4, Ou+P4) were pooled, log transformed and normalized, and compared by randomized complete block design (RCBD) with repeated measures. Values reported are the total pixel intensity (amount of Tyr-p).

	Good fertility animals				Low fertility animals			
Treatments	0 hr	3 hr	5 hr	SEM [†]	0 hr	3 hr	5 hr	SEM [†]
P4	-2507	^x -4830	^x 18957	4477	2778 ^a	-8529 ^a	83864 ^b	12630
Ouabain	-3279 ^a	^y 51967 ^b	^y 91908 ^b	6440	-10906 ^a	-14775 ^a	165744 ^b	16036
P4+Ouabain	9698 ^a	^{xy} 40426 ^{ab}	^{xy} 63502 ^b	5846	180 ^a	-2182 ^a	120154 ^b	14324

[†] Pooled standard error of mean.

^{a-b} Times within a treatment without a common letter differ, $P < 0.05$.

^{x-y} Treatments without a common letter differ at 3 and 5 hours for good fertility animals, $P < 0.05$. There were no significant differences at 0 hr for good fertility animals and at 0, 3 and 5 hours for low fertility animals